Role of endogenous H_2S in the attenuation of nickel-induced cell toxicity

By

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Abstract

Nickel as a heavy metal is known to bring threat to human health, and its exposure is associated with changes in fibroblast activation which may contribute to its fibrotic properties. H_2S has recently emerged as an important gasotransmitter involved in numerous cellular signal transduction and pathophysiological responses. Interaction of nickel and H_2S on fibroblast cell activation has not yet been studied. Here, we showed that low dose nickel (200 μM) induced the activation of human fibroblast cells, as evidenced by 15% increase in cell growth, increased migration and higher expression of α-smooth muscle actin (αSMA) and fibronectin, while high dose of nickel (1 mM) inhibited cell viability. We further found that nickel repressed the mRNA and protein expression of cystathionine gamma-lyase (CSE, a H_2S-generating enzyme) and blocked the endogenous production of H_2S. Exogenously applied NaHS (a H_2S donor) had no effect on nickel-induced cell viability but significantly attenuated nickel-stimulated cell migration and the expression of αSMA and fibronectin. In contrast, CSE deficiency deteriorated nickel-induced αSMA expression. Moreover, H_2S incubation reversed nickel-stimulated TGFβ1/SMAD1 signal and blocked TGFβ1-initiated expressions of αSMA and fibronectin. Nickel inhibited the binding of Sp1 with CSE promoter but strengthened the binding of Sp1 with TGFβ1 promoter, which was reversed by exogenously applied NaHS. These data reveal that H_2S protects from nickel-stimulated fibroblast activation and the CSE/H_2S system can be a potential target for the treatment of tissue fibrosis induced by heavy metal.

Keywords

Nickel, H_2S, Cystathionine gamma-lyase, Fibroblasts, TGFβ1

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Table of Contents

List of tables and figures

List of abbreviations

3-MST – 3-mercaptopyruvate sulfurtransferase

αSMA – Alpha smooth muscle actin

cDNA – Complimentary deoxyribonucleic acid

CAT – Cystathionine amino-transferase

CBS- Cystathionine β-synthase

ChIP – Chromatin immunoprecipitation

CO – Carbon monoxide

CSE – Cystathionine γ-lyase

DHLA – Dihydrolipoic acid

DMEM – Dulbecco's modified eagle medium

DPD – N,N- dimethyl-p-phenylenediamine sulfate

ECL – Enhanced chemiluminescence

ECM – Extracellular matrix

EDTA – Ethylenediamine tetraacetic acid

EMT – Epithelial-mesenchymal transition

FBS – Fetal bovine serum

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GSH – Glutathione

H2DCFDA – 2′,7′-dichlorodihydrofluorescein diacetate

H_2S – Hydrogen sulfide

HF – Human fibroblasts

KO – Knock out

MEF – Mouse embryonic fibroblast

MMP – Matrix metalloproteinases

mRNA – Messenger ribonucleic acid

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoli bromide

NaHS – Sodium hydrosulfide

NO – Nitric oxide

ORF – Open reading frame

P5P – Pyridoxal-5'-phosphate

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PPM – Parts per million

ROS – Reactive oxidative species

SDS-PAGE – Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sp1 – Specificity protein 1

TGFβ1 – Transforming growth factor β1

TRX – Thioredoxin

WT – Wild type

1 Introduction

1.1. Hydrogen sulfide (H_2S): the third gasotransmitter

1.1.1 H_2S sources and its effects

There are two main sources for environmental H_2S, organic and inorganic production. Organically produced H_2S comes from bacteria and organic decomposition, the main sites for this type of production include sewers, septic tanks and water treatment plants (1). Inorganically produced H_2S come from petroleum refineries, paper and pulp mills, and volcanos, etc (2). The human nose can sense H_2S at low concentrations of 0.3 ppm to 1.5 ppm, at this concentration H_2S is merely a scent nuisance (3). Clinical toxicological effects vary on length of exposure and concentration, H_2S is instantaneously fatal at concentrations above 500-1000 ppm. Prolonged exposure to concentrations between 10 and 500 ppm can cause sore throat, wheezing, shortness of breath, chest tightness, and hemoptysis (4). The mechanism of H_2S toxicity is related to its inhibition of oxidative phosphorylation (5). H_2S inhibits cytochrome *c* oxidase, the last enzyme in the mitochondrial respiratory electron transport chain in aerobic oxidative metabolism causing the cessation of ATP production (6).

1.1.2 H_2S as a novel gasotransmitter

Cellular signalling is often initiated by exogenous molecules binding to membrane receptors leading to intracellular messaging, there is a group of gases that are classified as exceptions (7). There are three gaseous signalling molecules identified thus far and they belong to the family

titled gasotransmitters (8). The three gasses are nitric oxide (NO), carbon monoxide (CO) and H_2S. Gasotransmitters are classified if they follow the six criteria in Table 1.1.2 (9).

Table 1.1.2 **Gasotransmitter criteria**

1. They are small molecules of gas.
2. They are freely permeable to membranes. As such, their effects will not rely on associated membrane receptors.
3. They are endogenously and enzymatically generated and their generation is regulated.
4. They have well-defined specific functions at physiologically relevant concentrations.
5. Functions can be imitated by exogenous products.
6. Their cellular effects may or may not be mediated by secondary messengers, but should have specific cellular and molecular targets.

H_2S is a colourless gas, which is flammable, lipid and water soluble and possesses a distinguishing rotten egg smell (10). H_2S has a vapor density of 1.19 which is greater than air. H_2S can be oxidized to form sulfates, elemental sulfur and sulfur dioxide (10). H_2S has a short half-life in the air that varies from 12 to 37 hours with variables including temperature, humidity and pollutants (11). Half-life within the body is mere minutes, and the metabolites include thiosulfate, sulfite, and sulfate (8). At pH 7.4, one third of H_2S remains undissociated while the remaining two thirds exist as anions (12). The following reaction (equation 1.1.2) represents the hydrolyzation of H_2S:

Equation 1.1.2

$$H_2S \rightleftarrows H^+ + HS^- \rightleftarrows 2H^+ + S^{2-}$$

1.1.3 Endogenous production of H_2S

Recent years have led to an overall accepted understanding of the endogenous H_2S production but research must continue to understand the H_2S signalling system and its role in disease

progression. One of the main biochemical means of H_2S signalling is through S-sulfhydration (13). S-sulfhydration is the post translational modification of protein that occurs when H_2S reacts with cysteine residues. The target proteins become modified into persulfide moieties (14). It is important to note that persulfide ligands have characteristics that are more favourable compared to thiols in the production of H_2S. Persulfides are metastable and decompose into smaller molecules, they have a higher reactivity compared to thiols as they better nucleophiles, reducing agents and they react with electrophiles. Persulfides can be produced enzymatically and non-enzymatically (14, 15).

H_2S is found at low concentrations of high nanomolar to low micromolar in circulation. H_2S is endogenously produced by three enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST) through two pathways, pyridoxal-5'-phosphate (P5P) co-factor dependent and P5P independent pathways (figure 1.1.3.1).

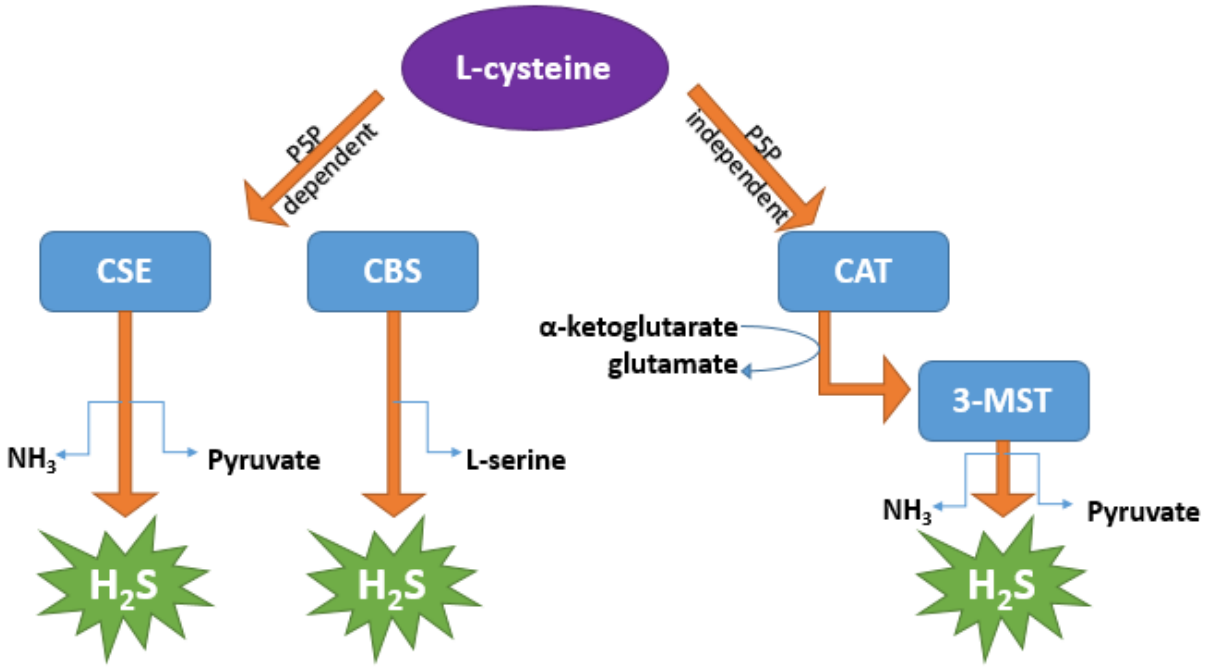

Figure 1.1.3.1 **Overview of H_2S producing enzymes**

CBS and CSE are localized in the cytoplasm while 3-MST is mainly localized to the mitochondrial matrix (16, 17). The production of the co-factor P5P comes from any of the 3 forms of vitamin B_6. The first most well-known pathway for endogenous H_2S production is P5P dependent, used both by CSE and CBS enzymes (18). H_2S production uses trans-sulfuration pathways, CBS is accountable for the first step of trans-sulfuration catalyzing homocysteine and serine to produce cystathionine (figure 1.1.3.2). CBS can also condense homocysteine and cysteine producing H_2S (figure 1.1.3.3).

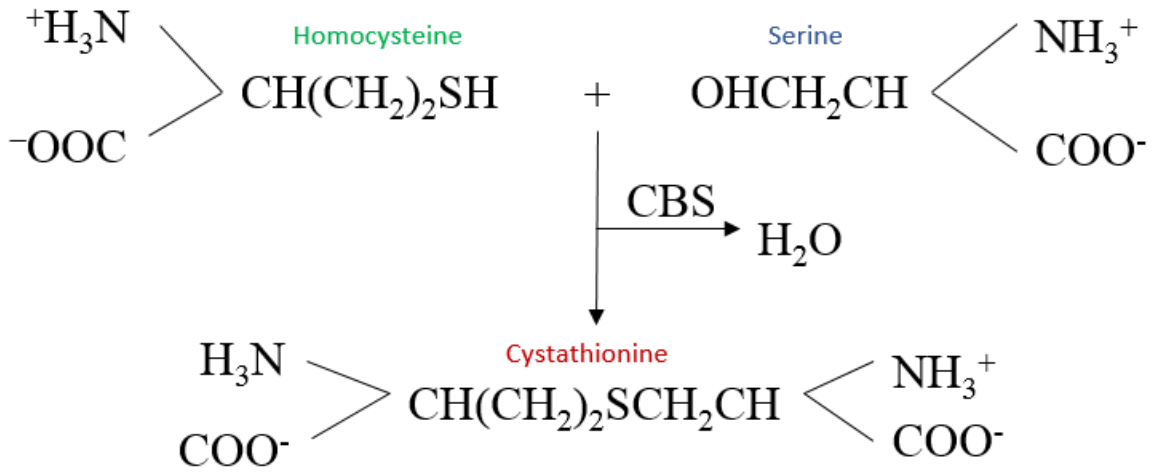

Figure 1.1.3.2 **First part of the transsulfuration pathway for homocysteine degradation**

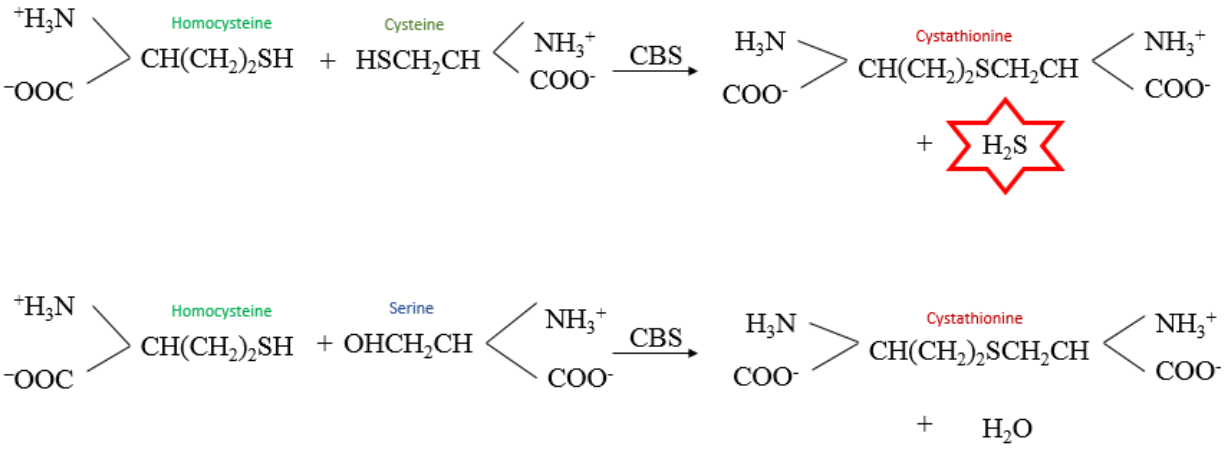

Figure 1.1.3.3 **CBS condenses homocysteine and cysteine or serine to produce H_2S**

The enzyme CSE is responsible for catalysing the second step of trans-sulfuration, cleaving cystathionine to produce L-cysteine, α-ketobutyrate and ammonia (figure 1.1.3.4) and then produce ammonium, pyruvate, serine and H_2S (19). The P5P independent pathway uses the cysteine aminotransferase (CAT)/3-MST pathway (figure 1.1.3.5) (20). CAT uses cysteine and α-ketoglutarate to first produce 3-mercaptopyruvate. 3-MST then requires endogenous reducing substances such as thioredoxin (TRX) and dihydrolipoic acid (DHLA) to release H_2S (19, 21).

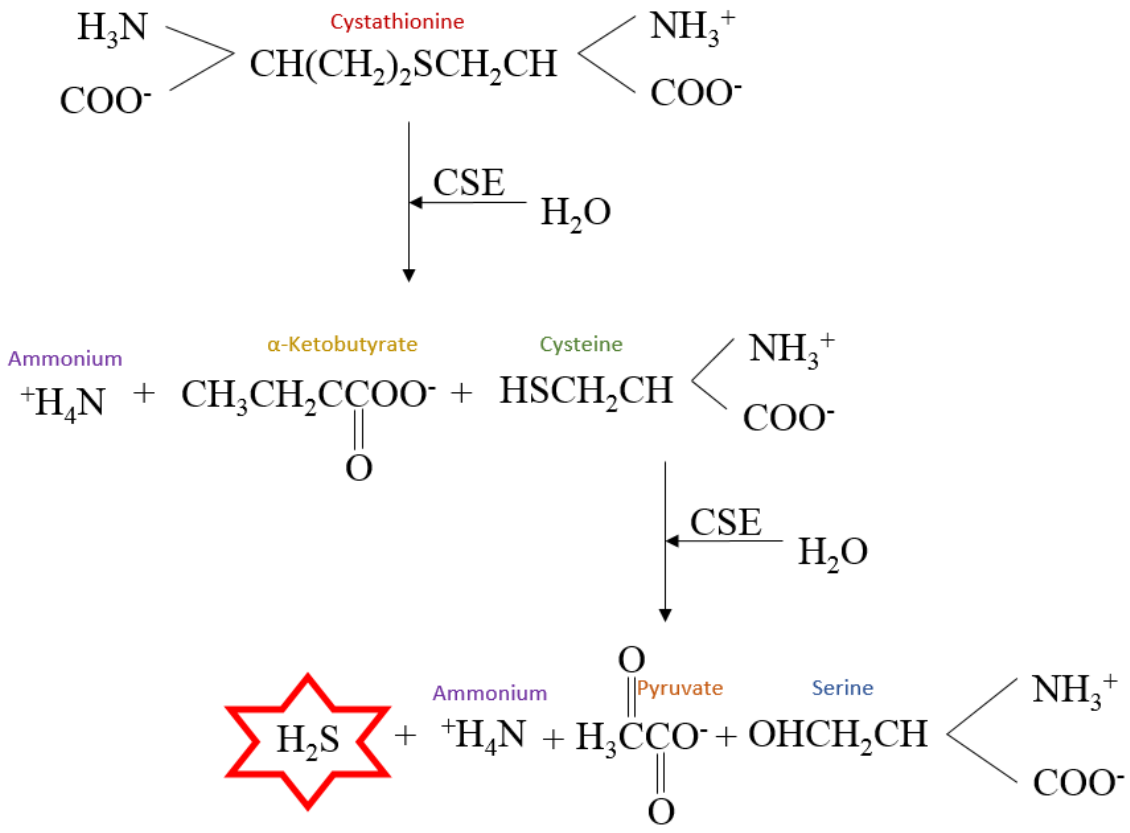

Figure 1.1.3.4 **Second part of the transsulfuration pathway for homocysteine degradation**

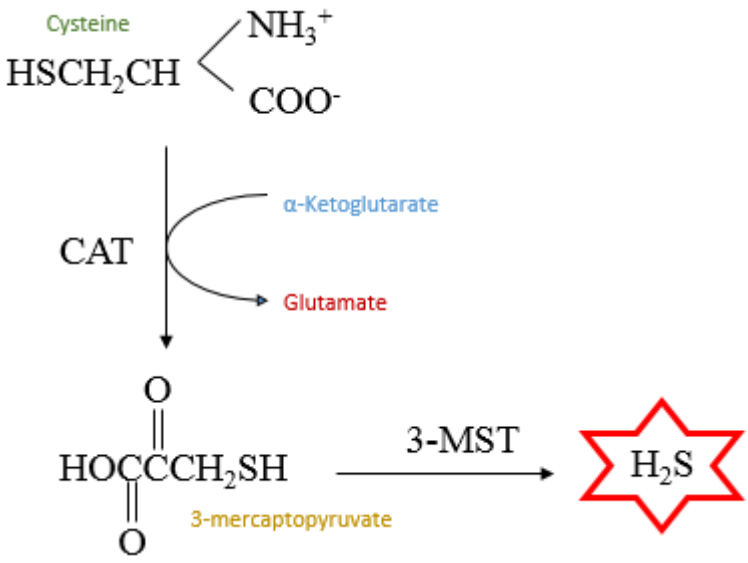

Figure 1.1.3.5 **P5P independent production of H_2S**

1.1.4 Implication of H_2S in both health and disease

Due to the current interest in understanding the roles of endogenous H_2S, research has determined several benefits to H_2S. There are three main biochemical means for direct H_2S signalling. It can modulate protein cysteine residues in the process of persulfidation, also named S-sulfhydration, H_2S can scavenge reactive oxygen species (ROS), and has the ability to react with proteins including proteins-containing metal centers (22, 23). Studies continue to scrutinize the role of H_2S in many aspects of physiology observing the means for H_2S direct interaction. Atherosclerosis, a leading cause of death in the developed world, is a chronic systemic disease that has been a focus in cardiovascular research (24). Many papers have shown the proper regulation of apoptosis has a defensive role in vascular protection in atherosclerosis disease states (25, 26). A study by Yang *et al.* demonstrated the important role of H_2S in cellular apoptosis (27). Although perhaps seeming counterproductive, apoptosis is in fact vital for normal

multicellular development, impairment of apoptosis can lead to vascular defects during embryonic development resulting in embryonic death as well as the role apoptosis plays in the pathology of atherosclerosis (28). Studies have concluded that H_2S induces apoptosis in human aortic smooth muscle cells through the activation of caspase-3 allowing normal vasculature development (28, 29).

In addition, the role of H_2S in cell proliferation has been observed; a study determined that H_2S has the ability to inhibit cell proliferation and DNA synthesis during H_2S overexpression. The study demonstrated that the enzyme producing H_2S induced cyclin-dependent kinase inhibitors leading to cell cycle arrest (27). When researchers notice anti-proliferative effects, it is logical to question the agent's role in cancer. A study looking at colon cancer observed that H_2S can offer beneficial antiproliferative action in a colon cancer cell line (30).

Another epidemic affecting western society is obesity, it is reported that obesity dramatically increases morbidity and mortality by increasing health complications such as cardiovascular disease, cancer, and type 2 diabetes (31, 32). A recent study, investigated the interaction of H_2S on the pathways contributing to the regulation of adipogenesis, where cells differentiate into mature adipocytes. H_2S was found to first increase differentiation, to cause changes to DNA binding and transcription factors, and to induce fat mass in both mouse and fly animal models (33).

The role that H_2S plays in diabetes has also been researched and it was found that H_2S is increased in pancreatic cells emulating a diabetic state. The increased H_2S production by enzymes causes a decrease in insulin levels (34, 35). A feedback loop begins as hyperglycemia inhibits H_2S producing enzymes (34). It is also found that H_2S can directly interact with a

glucose intermediate forming an H_2S-glucose adduct that is not recognized by transporters or receptors (36). Diabetic patients have increased risks of other diseases and complications that H_2S researchers have already focused on, including diabetic cardiomyopathy. The pathology of the disease includes myocardial hypertrophy, decreased cardiac contraction and an increase in extracellular matrix (ECM) proteins (37). Research in animal models focusing on the development of fibrosis, a hallmark of cardiomyopathy, have determined that H_2S has a protective effect at physiological concentrations on cells that differentiate into myoblasts. The main cause of this activation is due to cytokines signalling cell damage and the requirement to repair damaged tissue (38, 39). A study done by Meng *et al.* found the slow releasing H_2S molecule GYY4137 protects from myocardial fibrosis. Components of ECM and cellular proliferation were reduced by the treatment of exogenous GYY4137 via inhibiting the expression of transforming growth factor beta 1 (TGFβ1) and the SMAD pathway (40). This pathway is not yet fully understood and could be the key to understanding the protective role the H_2S system has in tissue damage.

1.1.5 H_2S regulation of protein by sulfhydration

A main cellular target of H_2S is the ATP-dependent potassium (K_{ATP}) channel, H_2S has been found to open the K_{ATP} channels specifically in vascular smooth muscles (41). The interaction of H_2S with K_{ATP} leads to vasodilation, protection from ischemia-reperfusion injury and myocardial injury (13, 42, 43). H_2S does not use a receptor to open the K_{ATP} channel but alters the channel by sulfhydration activating the channel by hyperpolarization (44, 45). The K_{ATP} channel is a heterooctomer comprising of equal numbers of pore-forming subunits (Kir6.x) and regulatory sulfonylurea receptors (SURx), at rest free thiols and disulfide bonds are at equilibrium and

when H_2S is added, disulfide bonds break in the SUR subunits altering its functionality (46, 47). This target could highlight an underlying mechanism for the regulation of cellular respiration by H_2S (48). In an in-depth review, Bibli *et al.* discussed H_2S's role in the cyclic guanosine monophosphate (cGMP) pathway. They found that fast releasing H_2S donors increased cGMP levels by inhibiting phosphodiesterase and by increasing NO bioavailability and affecting eNOS S-sulfhydration (49). Another pathway that is important in this research and how H_2S affects it, is the MAPK pathway (50). Many groups have determined that H_2S has the ability to protect endothelial and cardiovascular cells by inhibiting the p38, ERK and JNK pathways (30, 50-52). Inhibiting these MAPK family kinases protects cells against damage due to high level of ROS, and ischemia/reperfusion induced cell apoptosis (51, 52). Next, Guo *et al.* determined that H_2S has antioxidant effects by Keap1 S-sulfhydration leading to the activation of Nrf2 (51). Lastly, another target is the TGFβ receptor. In muscular and cardiac tissues, H_2S has been shown to prevent the activation of the TGFβ1/SMAD pathway and attenuates the production of ECM components (40, 53).

Proteins other than the three main H_2S producing enzymes have the ability to S-sulfhydrate as well, these include glyceraldehyde 3-phosphate dehydrogenase (GAPDH), protein tyrosine phosphatase (PTP1B), PTEN, Cu/Zn superoxide dismutase (SOD) for example (54-56). The "sensor" sulfhydryl group binds to sulfane sulfur to regulate their enzyme activity through hydropersulfide formation (21). A study found that approximately 10 to 25% of endogenous GAPDH are sulfhydrated *in vivo* and the sulfhydration happens at the Cys^{150} position, the cysteine that is vital to its catalytic activity, and thus changes the activity of GAPDH (56). The next example, PTP1B, a key component in regulating many signal transduction pathways, was found to be inactivated by H_2S through sulfhydration at the Cys^{215} position. A study determined

that the reaction occurs quickly, within two minutes of NaHS treatment (54). Another study tested PTEN as it is a main antagonist in a pathway responsible for the development of cancer. This protein is affected by sulfhydration at Cys^{124} after that, the protein undergoes a partial conversion with a disulfide bond between Cys^{124} and Cys^{71}, this change renders the protein inactive (55). Lastly the protein Cu/Zn SOD can undergo a change to form a persulfide group at the exposed Cys^{111}, this system is believed to be linked to primordial redox enzymes. The biological relevance of H_2S metabolism in the SOD system still needs to be clarified (57). The regulation of S-sulfhydration levels and therefore the regulation of H_2S is done by oxygen dependent catabolism through sulfide quinone oxidoreductase, sulfite oxidase and persulfide dioxygenase (58). The degradation of the persulfides is called desulfhydration and is assisted by a mitochondrial persulfide dioxygenase enzyme called ETHE1 (59).

1.1.6 Quick and slow H_2S releasing donors

To supplement the production of H_2S in laboratory settings, there are two types of H_2S donors that can be used, fast and slow releasing compounds. Examples of slow releasing compounds include stable molecules such as GYY4137 and S-diclofenac or fast releasing compounds that include sodium sulfide (Na_2S) or Lawesson's reagent (10). The most common donor used in H_2S related literature, even above H_2S-bubbled solution, is sodium hydrosulfide (NaHS) a fast releasing compound in water (equation 1.1.6), this choice is due to reasons including the convenient preparation without the strong smell and the forward dissociation at relevant pH of ~7.4.

Equation 1.1.6

$$NaHS \rightleftarrows Na^+ + HS^-$$

$$HS^- + H_2O \rightleftarrows H_2S + OH^-$$

In conclusion, H_2S was originally seen as a dangerous and toxic gas, as the study of H_2S has progressed, H_2S has been shown to have roles in anti-inflammation, cardio-protection, neuro-protection, cytoprotection, as well as protection against oxidative stress (46, 60-63). Studies by groups like Zheng *et al*. have further shown cardiovascular benefits of H_2S. They determined exogenous H_2S will stop fibrotic response induced by hyperglycemia in cardiac fibroblasts (38). With the determination of positive protective abilities, it is important to further explore the beneficial effect of H_2S, especially to further H_2S research and observe other potential protective effects, including its protection against trace metals.

1.2 Fibroblasts

1.2.1 Fibroblast cell activation and fibrosis

Fibroblast cells can be found all over the body, especially in the connective tissue, which connects and supports all tissues in the body. Fibroblast cells are normally quiescent with less proliferative and migratory property (64). In response to cytokines or injury, fibroblast cells can be activated from its quiescent form to myofibroblasts, which start to proliferate, migrate, and secrete extracellular matrix proteins (ECM) (65). It is important to note that healthy normal tissue rarely comprise a population of myofibroblast cells (66). ECM contains components of structural proteins, ground substances, and adhesive proteins, including collagen, glycoproteins, and fibronectin, etc. (67). The activation of fibroblasts to myofibroblasts permits the contraction of stress fibers through ECM to close and heal a tissue wound (66). Over-activation of fibroblasts will lead to tissue fibrosis. Other than fibroblast cells, smooth muscle cells, epithelial cells,

endothelial cells, fibrocytes and pericytes can also differentiate into myofibroblasts and contribute to tissue fibrosis (67).

Fibroblasts have a few biological roles including wound healing, inflammation, angiogenesis and paracrine and autocrine signaling (68). Wound healing includes proliferation through chemotaxing, the activation of fibroblasts inducing cell contractions allow for wound closures, and can promote blood clotting (68, 69). Fibroblasts allow the accumulation of immune and inflammatory cells through the ground substance of ECM to the injury site. Fibroblasts secrete TGFβ1, some interleukins (IL), and ROS, they also help activate macrophages (70, 71). Angiogenesis that is beyond the reach of established blood vessels is essential and the ground substance of the ECM produced by fibroblast allows endothelial cells to migrate and build vessels (72). There are paracrine signaling molecules that act on fibroblasts released by myoblast precursor cells to induce ECM production, proliferation and differentiation. These molecules include platelet derived growth factor, IL-6 and IL-13 (73). IL-13 promotes TGFβ activation, and production along with other ECM components (74). After the initial paracrine signals onto fibroblast, the cells then produce paracrine signals, these include connective tissue growth factors and ROS further contributing to fibrotic responses (75). TGFβ is a signalling molecule that acts upon and is generated by fibroblasts in the differentiation to myofibroblasts, a few ILs have this quality as well (70, 73). Excessive activation or prolonged response to cellular injury leads to the pathological process of fibrosis causing scarring and eventual organ hardening and deformation, impairing organ function ultimately ending with organ failure leading to the advancement of many diseases (76).

1.2.2 TGFβ1/SMAD signalling and fibroblast activation

Fibroblasts require chemical stimulation to differentiate into myofibroblasts, the activated cell will then secrete autocrine agents like TGFβ1 (73) and paracrine agents like ROS (75). TGFβ is one of the most potent pro-fibrotic cytokines involved in the differentiation of fibroblasts. There are three isoforms of the TGFβ protein, TGFβ 1, 2, and 3, that share three quarters homology in amino acid sequence. The protein TGFβ is a six cysteine knot motif with three disulfide bonds that stabilize β-sheets (77). TGFβ ligands are secreted from the fibroblasts in their latent precursor form; they are activated by proteolytical cleavage of the latency-associated peptide in the ECM. The activation of TGFβ involves conformational change by cleavage, physical interaction with other protein or endoglycosylases (78).

Signal transduction in the TGFβ1/SMAD pathway begins with initial binding to a serine threonine kinase receptor. The receptor contains a cysteine rich extracellular region and kinase domains intracellularly (79). TGFβ binds to the type II receptor at the cell membrane, TGFβ1 receptor would next be phosphorylated leading to the activation of kinase activity. The cascade will then lead to intracellular signalling (80). The SMAD proteins allow signalling from the receptor to the nucleus by phosphorylation of the SMAD family. Some SMAD proteins bind to DNA with simply a 5 base pair sequence recognition, while others do not bind to DNA directly (81). A group of SMAD components are actually inhibitory SMADs preventing the SMAD signalling by translocating into the nucleus and causing ubiquitination (82). The TGFβ1/SMAD signalling pathway is significant and it was found by cDNA/promoter testing that TGFβ1 acts upon sixty ECM related genes (83). The TGFβ1 promoter could be acting on the interaction between SMAD and specificity protein 1 (Sp1) (84). Sp1 is a zinc finger transcription factor that preferentially binds to GC-rich motifs. Studies have proved Sp1 plays a role in TGFβ1 and H_2S

producing CSE genes (85, 86). Sp1 is important in the expression of ECM components like collagen, cytokines like TGFβ1 and interacts with SMAD, this makes it a target that interferes with pro-fibrotic activity and a factor to observe in the research of fibrosis (85).

Exogenous TGFβ1 has the ability to improve wound healing; some research has shown that perhaps the SMAD pathway works by suppressing the inhibitory SMADs. In a study with SMAD inhibitors knocked out, exogenous TGFβ1 reversed the ECM effect indicating perhaps TGFβ1 works with a SMAD independent pathway (77). Thus the effects of TGFβ is mediated by intracellular signalling that include SMAD-dependent and SMAD-independent pathways (figure 1.2.2). The independent pathway include mitogen activated protein kinases (MAPK) that have a role in cytokine and ECM gene expressions, migration and apoptosis (87).

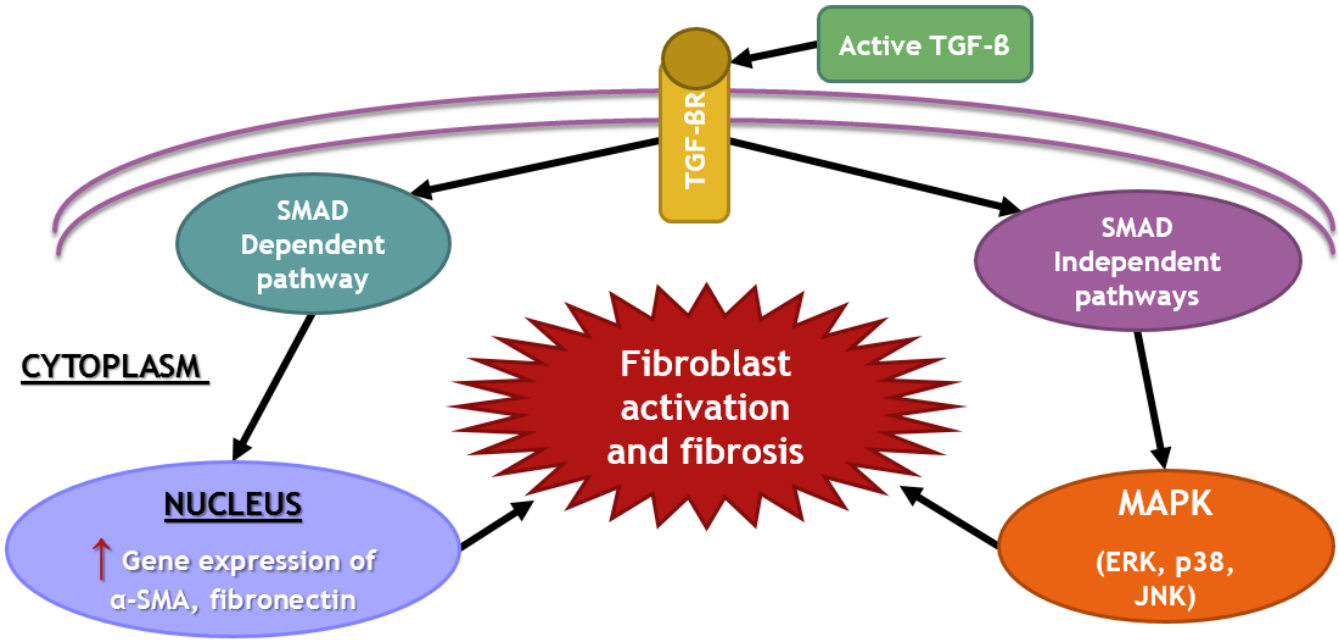

Figure 1.2.2 **TGFβ activating on fibrotic pathways**

The TGFβ1/SMAD pathway is also involved in cross signalling mechanisms as some SMAD components have been seen to affect vitamin D3 induced transcription. Vitamin D receptors play

a role in cell proliferation and differentiation, apoptosis, and oxidative stress regulation (88). SMAD interacts with many transcript factors or co-factors like CBP/p300 by exposing the underlying DNA sequence to transcripts. Its interaction with Sp1 is likely a major reason for the role SMAD plays in transcriptional cross-talks, Sp1 is responsible for the transcription of many genes and the effects are dependent of the target promotors structure (89). The TGFβ1/SMAD pathway will induce targeted fibrotic gene expression of ECM components and therefore prove to be an important pathway in the understanding and prevention or protection of fibrotic diseases (77, 90).

1.2.3 MAPK pathway and fibroblast cell activation

The MAPK family is a group of threonine and serine protein kinases that play a role in signal transductions affecting gene expression. This family is separated into supplementary groups, the conventional sub-groupings are termed extracellular signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 (91). In mammals, fourteen MAPK proteins have been characterized, these are generally activated through phosphorylation and/or by the interaction with a small binding protein. The conventional MAPKs are composed of a set of sequential kinases: MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (92). MAPKs respond to stress inducements or cytokines such as TGFβ1 and regulate differentiation, proliferation, cell survival, and apoptosis, all key factors in fibrosis progression (93). MAPK is collectively known to act as a downstream target of TGFβ1, inhibiting MAPK could prove to be a target for the progression of fibrosis (94).

1.2.4 αSMA and fibroblast cell activation

Quiescent fibroblast cells are characteristically elongated and flat spindle-like shaped. Myofibroblasts are characterized as having a starburst shape, expressing alpha-smooth muscle actin (α-SMA), being contractile, producing and secreting ECM at a higher rate and becoming resistant to apoptosis (95). The actin fibers were originally thought to bundle and surround the wound in tissue injury as the first phase of healing followed by differentiation and proliferation of myofibroblasts (96). Later it was isometric tension testing in a collagen gel that highlighted contractile forces by α-SMA and ECM after wounding (97). ECM contains fibronectin, a protein that assists in the formation of connections between cells through integrin proteins (98), collagen, providing structural scaffolds for tissue regeneration (99) and α-SMA, fibers that function as contractile devices essential in cell migration (73). The ground substance of ECM is a proteoglycan gel allowing for nutrient flow past the reach of blood vessels, it also facilitates intercellular signalling and cell migration (67).

1.2.5 MMP and fibroblast activation

Fibroblasts are responsible for the breakdown and reabsorption of the produced ECM by matrix metalloproteinases (MMP), a protein that breaks down ECM to inhibit fibrotic effects. In contrast, other tissues such as bones have individual cells: osteoblasts to produce bones and osteoclasts that reabsorbs bone (67, 100). MMPs are a family of over 24 zinc-dependent endopeptidases that have the ability to degrading nearly all components of the ECM. Their ability to act upon ECM components make them vital in the process of tissue remolding (101). MMPs are expressed in enzymatically inactive states due to an interaction between a cysteine residue of the pro-domain with a zinc ion of the catalytic site. Only once this link is broken do

the MMPs become active (102). MMP-2 is one of the most common proteinases in the process of controlling ECM. Excessive levels of MMPs can damage the ECM network and affect essential tissue remodeling and signaling, a reduced level of MMPs would lead to accelerated progression of fibrosis (103).

1.2.6 Treatment of tissue fibrosis

Organs typically affected by fibrosis include the liver, kidney, heart, lungs, gastrointestinal tract and skin. These organs are altered due to different factors including autoimmune disorders, old-age through progressive fibrosis or exposure to toxic agents (104, 105). There exists some therapies targeting fibroblast in treatments for fibrosis but with so many factors affecting myofibroblast differentiation, the single target treatments prove to be less effective than desired. For example an inhibitor of angiogenesis, endostatin, can act on fibroblasts but was originally clinically tried to be an anti-cancer agent acting on endothelial cells. Endostatins are peptides about 20 kDa, fragmented from the C terminus of collagen XVIII (106). A study by Yamaguchi and associates concluded that endostatins prevented and reversed the effects of TGFβ therefore suppressing the excessive production of ECM. The endostatin levels in serum are elevated and the groups hypothesized a negative feedback regulatory loop in preventing the progression of fibrosis (106, 107). Fibrocyte receptors are another therapeutic target that show potential in fibrosis treatments, the CXCR4 receptor is responsible for the facilitation of cell migration. The receptor is inhibited by caveolin 1, a caveolin peptide. It impedes the buildup of fibrocytes in the lungs of an animal model (108).

1.3 Metals

1.3.1 Metal in human health

The interaction of H_2S with trace metals is also an increasingly researched topic and proves to be an interesting relationship to explore. Metals can be found almost everywhere in daily life and the overuse of the metals have had a huge impact in human health. Some metals at specific low concentrations like copper, iron, magnesium, nickel and zinc are regarded as essential nutrients for the body's daily functions. Increasing the concentration of any of these trace metals significantly will lead to cell damage and disease. Industrial development and smelter related industries are the main sources of exposure of metal to the environment (109, 110).

1.3.2 Nickel in human health

Nickel, one of the trace metals, is mined mainly from sulfide ores and undergo processes involving fusion, heat and electrolysis for purification. One of the top countries that produce nickel in the world is Canada, with a large deposit located in the Sudbury basin in Ontario (111, 112). Nickel is also widely found in air pollution, diesel exhaust or in a variety of industrial and consumer products, such as metal household items, jewellery, stainless steel, coins, cigarettes, and batteries, etc. In the clinical setting, nickel can be often found in the implantations of a metal stent or the use of nickel-chromium alloy occluder for treatments of cardiovascular disorders (109, 111, 113). As the population continues to have long term contact with nickel-containing consumer and clinical products, it is important to understand its risks to human health. Nickel is resistant to corrosion from water and air but easily dissolved in oxidizing acids (114). Nickel has an atomic number of 28 and is a transition metal with oxidation numbers of -1, 0, +1, +2, +3 and

+4 in compounds with Ni^{2+} being the most common. Nickel can be found in different forms that include elemental nickel, nickel oxide, nickel chloride, nickel sulfate, and nickel monosulfide (115, 116).

Prolonged exposure to nickel by means of inhalation, ingestion or through dermal absorption can cause contact dermatitis, immunotoxicity, pulmonarytoxicity, neurotoxicity, genotoxicity, and carcinogenicity (117, 118). Many of the harmful effects of nickel arise from its interference with essential metal metabolisms such as zinc, magnesium or iron (114). Nickel crosses into the cell membrane through calcium channels and will compete for specific receptors with calcium (119). In metal-dependent enzymes, nickel can substitute for other trace metals, particularly zinc, altering the protein and ultimately the protein activity or function, this is seen in the Sp1 zinc finger transcription factor (118, 120). Nickel interacts with amino acids to form nickel complexes with cysteine, histidine and aspartic acid by its ability to bind to oxygen, sulfur and nitrogen side groups. Nickel has been found to have a better binding affinity for proteins compared to DNA, the affinity of DNA phosphate groups with nickel is less than that of the amino acid functional groups. Nickel in the cell is able to induce conformational transitions in oligodeoxynucleotides from the right-handed B helix to the left-handed Z helix (121). Previous studies have determined that nickel can induce DNA-protein crosslinks that will lead to the formation of ROS (120, 122). Nickel is excreted from the body through excrements and urine depending of the exposure route (121). Absorbed nickel is filtered in the kidney through the glomerulus and then low molecular weight nickel compounds are successively reabsorbed through capillary (123).

Some organ systems are more sensitive to nickel toxicity including the respiratory system, cardiovascular system, and the kidneys. First the nose and throat area can become irritated,

especially in people suffering from asthma. Individuals with lung lesions or diseases can worsen their symptoms through inhaled pollution containing nickel (124). In the cardiovascular system, nickel can cause anemia and increase blood pressure (125). Exposure to nickel induces kidney damage, increasing the formation of stones and leading to renal cancer (126). Nickel may also act directly on lipids and proteins as pro-oxidants or even as free radial generators. ROS are known to harm proteins, lipids, DNA and interfere with cellular signaling pathways. A schematic example of oxidant-mediated lung dysfunction due to higher level of nickel can be seen in figure 1.3.2 (127).

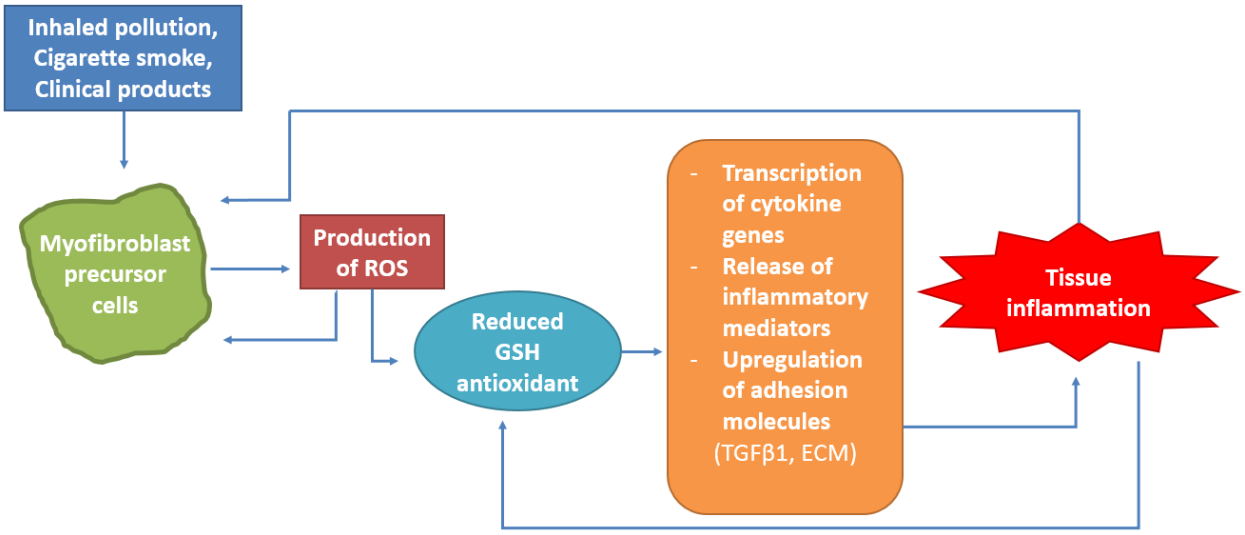

Figure 1.3.2 **Oxidant-mediated inflammation**

1.3.3 H_2S interacting with metals

Sulfides have been used in histochemistry for over a century and a half, they transform the available metals like copper, iron, zinc, nickel, mercury, lead and arsenic in tissue to metal sulfites. An example of this type of histochemistry is the sulfide silver method testing where the

sulfites catalyze the reduction of silver ions binding the silver in place, permitting the analysis of these trace metals (128). Metalloproteins utilize oxygen, nitrogen and or sulfur from amino acid side chains like cysteine to bind the metal ion tightly, other more interactive protein ions can be generalized as highly exchangeable, mobile or labile (129, 130). For example zinc ions are mostly considered to be signalling molecules in inter/intra-cellular signalling therefore, a lot of labile zinc can be found inside the cells. The relationship sulfur has with zinc is evident when looking at zinc finger proteins where two of the four binding ligands are cysteines (131). Thiol ligands give zinc the ability to be thermodynamically stable while allowing fast ligand exchange through reversible redox reactions. A useful example of this dynamic is the interaction of zinc with metallothioneins, cysteine rich low molecular weight proteins. Zinc would be released from metallothioneins and transferred to sulfhydryl groups on oxidation susceptible proteins during oxidative stress to avoid oxidation (132). Metallothioneins also have the ability to cluster copper, maintaining biological metal homeostasis and providing protection from harmful molecules such as toxic heavy metals including mercury, lead and cadmium. Insufficient copper can lead to defective copper containing enzymes such as cytochrome *c* oxidase, dopamine hydroxylase or tyrosinase (133, 134). The contents and role of the metallothionein cluster depend on three factors, i) the source of the protein, ii) any previous exposure to metal ions, and iii) the primary sequence of the protein chain as it can partially select for specific metals (134). From toxic metals to metal ions required for normal function, sulfides have a natural function in the clearing or the transport of metal ions.

Concentrating on nickel now, much research has been conducted with regards to nickel and sulfur's chemical relationship. Much of the research focuses on H_2S gas sensing and detection as a way to protect against the toxic effects of H_2S (135, 136). Lee *et al.* discovered that sensors

promoted with nickel oxide were able to increase sensitivity down to 1 ppm with a high sensor response percentage. In a different type of study, nickel sulfide was found to improve structural stability during repeated sodium insertion and extraction processes in sodium-ion batteries (137). Exploring the role of nickel and H_2S in cellular functions is innovative and novel, research has focused on the toxic effects of nickel at high concentrations in cells while pathophysiological concentrations and their effects with endogenous H_2S has yet to be examined.

2 Hypothesis and objectives

Nickel has been shown to induce the activation of fibroblast cells and lead to tissue fibrosis (138, 139) and the beneficial roles of H_2S in the progression of various tissue fibrosis have also been documented (140-142). However, the cross talk between nickel and endogenous H_2S signaling system in fibroblast cell activation has not been investigated. Here it is hypothesized that endogenous H_2S may protect nickel-induced fibroblast cell activation. To this end, two objectives are proposed: 1) to determine the effect of nickel on H_2S signalling in human fibroblast cells; 2) to investigate the mediation of endogenous H_2S signalling on nickel-induced fibroblast cell activation as well as the underlying mechanism. This project would provide an important potential target for the treatment of tissue fibrosis.

3 Methodology

3.1 Cell culture and treatments

Human fibroblast (HF) cells from skin tissue was purchased from American Type Culture Collection (Manassas, VA, USA). Experiments were conducted using (6-12) passages. The cells were recovered and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Oakville, ON, CA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher, USA) and 1% penicillin–streptomycin solution (Hyclone, Thermo Fisher, USA). The cells were kept in an incubator at 37 °C in a 5% CO_2, 95% air atmosphere.

Mouse embryonic fibroblast (MEF) cells were isolated from the embryos in both CSE knock out mice (MEF-KO) wild type littermates (MEF-WT) as described previously (143). The cells were maintained in DMEM supplemented with 20% FBS and 1% penicillin–streptomycin solution, and (3-6) passages were used in this study.

3.2 Cell viability and cell number

Equal numbers of cells were seeded and grown to approximately 80% confluence and a gradient of treatments were incubated for 24 hours in 96 well plates. Briefly, the treatment was washed away by PBS and 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) was added to each well and incubated at 37 °C for 4 hours. The reduction of the yellow MTT to dark blue/purple formazan, is done in the mitochondria, thus by living cells only (144). Formazan can be dissolved in DMSO, the solution was then read spectrophotometrically. The absorbance was

measured using a FLUOstar OPTIMA microplate spectrophotometer (BMG LABtech, Germany) at 570 nm. Cell viability was expressed as a percentage of untreated control cells.

To further confirm the effects of cell viability, cells were removed by trypsinization and resuspended for cell number counting using a hemocytometer.

3.3 Cell migration

The cell's ability to repair, regenerate and migrate was analyzed by scratch wound assay (145). Briefly medium was removed from post-confluent HF cells and they were subjected to a single pass with a medium sterile pipette tip causing a "wound". Serum free medium and the treatments groups were added for 24 hours. Wound closure was monitored and pictures were taken using Olympus IX71 microscope and DP2-BSW computer software at times 0 and 24 hours. The migrated area of the cells was measured and analysed using Image J 1.43v software.

3.4 Western blot

Protein expression levels were tested by western blotting. After treatments, HF and MEF cells were collected and lysed in tris EDTA with sucrose buffer along with protease inhibitor (Sigma-Aldrich, Oakville, ON, CA). The supernatant comprising cellular proteins was collected following centrifugation at 15 000 × g for 15 minutes. Proteins were denatured with heat and extracts were separated by a 10% SDS-PAGE and blotted onto nitrocellulose membranes (Pall Corporation, Pensacola, FL, USA) as previously described (146). Membranes were blocked in 3% milk in PBS, next the membranes were probed with appropriate primary antibodies and detected using peroxidase-conjugated secondary antibodies 1:5000 (Sigma-Aldrich, Oakville,

ON, CA) and finally the signals were visualized by ECL (GE Healthcare, Amersham, UK). The dilutions of primary antibodies were: 1:5000 for β-actin (Sigma-Aldrich, Oakville, ON, CA), 1:1000 for αSMA (Abnova, Taiwan), 1:1000 for CSE (Abnova, Taiwan), 1:1000 for CBS (Abnova, Taiwan), 1:1000 for 3-MST (Abnova, Taiwan), 1:200 for Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA), and 1:200 for MMP2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:200 for TGFβ1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:1000 for SMAD1, SMAD2 and SMAD3 (Cell Signaling Technology Inc., Whitby, ON, CA). Bands were always normalized against the β-actin band.

3.5 RNA extraction, reverse transcription and real-time PCR

After treatments, the cells were collected and RNA was extracted using TRIzol reagent (Sigma-Aldrich, Oakville, ON, CA). Following centrifuge at 12 000 × g for 15 minutes, the clear upper phase of the samples was transferred to new tubes and precipitated with isopropanol. Total RNA was reverse transcribed into cDNA using random hexamer primers according to manufacturer's protocol (Thermo Fisher Scientific, Ottawa, ON, CA). Negative controls were prepared to ensure no DNA contamination was present (147). The quantification of mRNA expression was performed with an iCycler iQ5 apparatus (Bio-Rad, Mississauga, ON, CA) associated with the iCycler optical system software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad). The sequences of primers were: αSMA (5'-CCACTGCCGCATCCTCATCCT-3' and 5'-CCCGGCTTCATCGTATTCCTGTT-3'); fibronectin (5'-GGCTGCCCACGAGGAAATCT-3' and 5'-GCCACGGCCATAGCAGTAGCAC-3'); CSE (5'-CCCCCATCTCACTGTCCACCAC-3' and 5'-CAGCCTTCAATGTCAATCACCTTC-3'); and GAPDH (5'-GCGGGGCTCTCCAGAACATCAT-3' and 5'-CCAGCCCCAGCGTCAAAGGTG-3').

The cycling condition template started at 95°C followed by 38 cycles consisting of 95°C for 15 seconds and 60°C for 60 seconds. A standard melting curve was performed by increasing the temperature to 95°C for 60 seconds, decreasing the temperature to 55°C and increasing the temperature by 1°C. Relative mRNA quantification was then calculated by using the formula $2^{-\Delta\Delta CT}$, where ΔCT is the difference between the threshold cycle of a given target cDNA and an endogenous reference of GAPDH gene.

3.6 Thiol detection through fluorescence

The cellular thiol contents was analyzed by a thiol detection assay kit (Cayman chemical, Ann Arbor, MI, USA). Briefly, equal amounts of protein was added into the well containing 50 µL thiol fluorometric detector. The plate was then incubated in the dark for 5 minutes. After that, the plate was read at excitation wavelength of 340 nm and an emissions wavelength of 520 nm using a FLUOstar OPTIMA reader and OPTIMA software (version 2.00R3).

3.7 ROS detection

After different treatment, the cells were first rinsed with PBS twice then incubated with 1 µg/mL of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen, Carlsbad, CA, USA) a fluorescence probe to visualize the presence of ROS in the cells (148). Images were obtained 30 mins after the addition using Olympus IX71 microscope and DP2-BSW software. Image J 1.43 software was used to quantify fluorescent intensity.

3.8 Immunofluorescence staining

HF cells were grown, treated and then fixed with a 4% paraformaldehyde/PBS solution for 15 minutes, the cells were permeabilized in a 50% acetone: 50% methanol solution after fixing. These cells were stained with anti-α-SMA antibody (Abnova, Taipei, 1:500) followed by FITC-conjugated secondary antibody (Sigma-Aldrich, Oakville, ON, CA, 1:2000) (86). The immunoreactions were visualized though Olympus IX71 microscope and DP2-BSW software.

3.9 Measurement of endogenous H_2S production

WT mouse liver and HF cell lysate were used for analysis of H_2S production rate. The production rate was measured with a methyl blue method as previously described (41, 149). The flasks were mixed with 100 mM potassium phosphate buffer, 10 mM L-cysteine, 2 mM pyridoxal 5-phosphate, 10% (w/v) liver lysate, and various agents as indicated in the figures. Next, a center well containing 0.5 mL 1% zinc acetate and a 2 cm × 2.5 cm piece of filter paper were added to the reaction flask. The flasks were flushed with N_2, sealed with Parafilm and then incubated at 37°C for 90 mins. The reaction was stopped by adding 50% trichloroacetic acid then the flasks were incubated again at 37°C for 60 mins. The contents of the center wells were transferred to test tubes containing 3.5 mL of water. Then, 0.5 mL of both 20 mM N,N-dimethyl-p-phenylenediamine sulfate in 7.2 M HCl and 30 mM FeCl3 in 1.2 M HCl were added to the test tube to produce methylene blue. The test tubes were incubated at room temperature for 20 mins and the solution was measured spectrophotometrically at 670 nm using a FLUOstar OPTIMA microplate spectrophotometer. The amount of H_2S was calculated against an H_2S standard curve and expressed in nmole/g/min.

3.10 H_2S direct chemical interaction

The direct interaction of H_2S with $NiCl_2$, fibronectin, collagen and TGFβ1 was analyzed with a lead sulfur method (150). The solution containing NaHS with or without $NiCl_2$, fibronectin, collagen and TGFβ1 at the concentrations as indicated in the figures was placed in a 96-well plate. Lead acetate paper (Sigma-Aldrich, Oakville, ON, CA) was secured above the liquid phase contained in the 96-well plate with a cover. The reaction was incubated for 1 hour at 37°C in the dark. H_2S gas was released and reacted with the paper forming yellow-brown color. The well only containing NaHS acted as the control. The lead acetate paper was then scanned and the colour change was analyzed with Image J software.

3.11 Sp1 gene transfection

Sp1 gene was transfected into HF as described previously (86). Briefly 1 μg of pcDNA3.1-Sp1 vector or pcDNA3.1-control vector was transfected into HFs with lipofectamine 2000 (Invitrogen Carlsbad, CA, USA). The cells were incubated with the vectors for a total for 24 hours therefore 0.2 mM $NiCl_2$ was added for additional 24 hours. The cells were then harvested and lysed, and the protein expressions of Sp1, CSE, and TGFβ1 were measured with western blotting.

3.12 Chromatin immunoprecipitation (ChIP)

To determine the direct interaction of nickel on Sp1 binding with the promoters of CSE and TGFβ1, a ChIP assay was conducted. After different treatments, the cells were treated with paraformaldehyde to crosslink protein and DNA interactions and nuclear material was extracted

from the cells. Next the antibody against Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to precipitate Sp1 antigen. After purification, the Sp1 protein was then removed by protease leaving only the DNA fragments that was bound to Sp1 transcription factor (151). The intensity of Sp1-binding DNA region was then determined by real-time PCR. PCR primers 5′-GGACTCCAGCTTCACTCCGCTT-3′ (forward) and 5′- TTAGCGGGTCTGCAGTCTCACG-3′ (reverse) were used to amplify Sp1 binding site in human CSE promoter (86). PCR primers 5′-CCCCGCGAGGAGGCAGGAC-3′ (forward) and 5′-TCAGGGAGAAGGGCGCAGTGGTG-3′ (reverse) were used to amplify Sp1 binding site in human TGFβ1 promoter. The binding intensity of Sp1 with gene promoters was normalized to the level of input. Input is the nuclear material that was extracted from the cell without the addition of the antibody in the purification step.

3.13 Fluorescent detection of labile zinc

FluoZin-3-AM (Invitrogen, Carlsbad, CA, USA) is a specific fluorometric detector for visualization of labile zinc (152). After different treatments, HF cells were washed three times with PBS and incubated with 1 μM FlouZin-3-AM for 1 hour and 24 hours at 37°C. The cells were then rinsed three times with PBS, the fluorescence signals were detected using an inverted Olympus IX70 fluorescence microscope. Then, Image J 1.43 software was used to quantify fluorescent intensity.

3.14 Statistical analysis

All results were presented as means ± SEM, representing at least 3 independent experiments. Statistical comparisons were made using two-tailed Student's *t*-tests or one-way ANOVA followed by a post-hoc analysis (Tukey test) where applicable. Values of $p<0.05$ were considered to be statistically significant.

4 Results

4.1 H_2S blocks nickel-stimulated cell migration

The effect of increasing concentrations of nickel on HF viability was first determined. As illustrated in figure 4.1.1, nickel at concentration higher than 1 mM started to inhibit cell viability, and 5 mM nickel reduced cell viability by more than 80%. However, the cell viability was increased by nickel at low concentration. In comparison with the control cells, nickel at 0.1 mM and 0.2 mM significantly induced cell viability by 16% and 15%, respectively. Since this study focuses on effect of nickel in fibroblast cell activation at low doses, 0.2 mM $NiCl_2$ was chosen in later experiments. We next evaluated the interaction of nickel and H_2S on cell viability, cell number and cell migration. NaHS is used as a quick H_2S-releasing donor, when dissolved in solution at pH 7.0, about one third NaHS becomes H_2S gas. In the present study, anhydrous NaHS at 30 μM was added into the culture medium, which releases about 10 μM H_2S within hours. The endogenous H_2S concentration has been reported to high nano-molar to low micro-mole range, the concentration of H_2S used here is physiological relevant. The supplement of NaHS (30 μM) had no effect on nickel (0.2 mM)-induced cell viability and cell number (figure 4.1.2 and figure 4.1.3); however NaHS significantly inhibited nickel (0.2 mM)-stimulated cell migration (figure 4.1.4). NaHS (30 μM) alone had no effect on cell viability, cell number and cell migration.

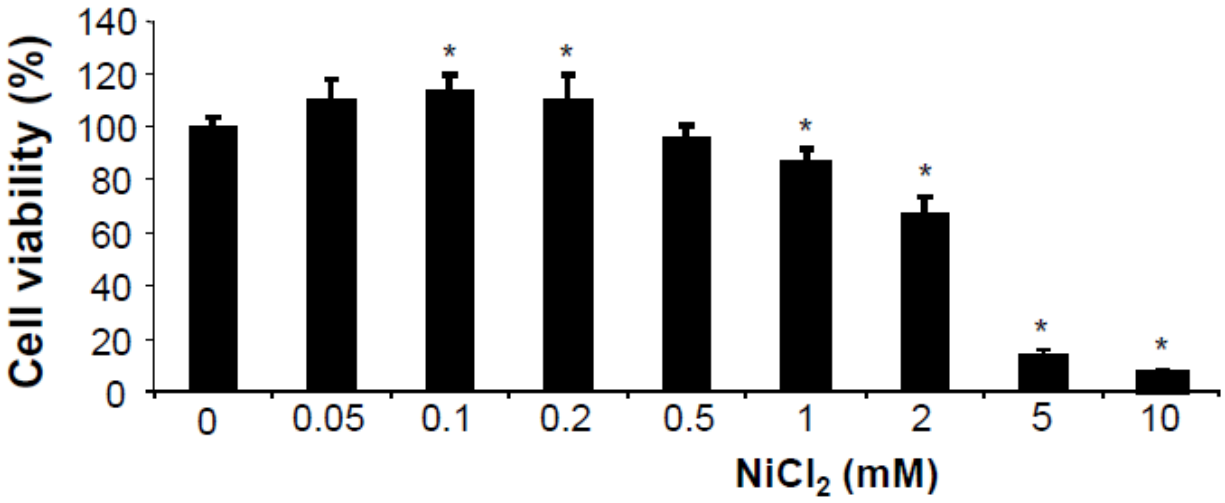

Figure 4.1.1 **Effect of nickel on HF cell viability.** HF cells were incubated with $NiCl_2$ at the indicated concentrations for 24 hours. Cell viability was measured following treatments by MTT assay. * $p<0.05$ versus control. n =4

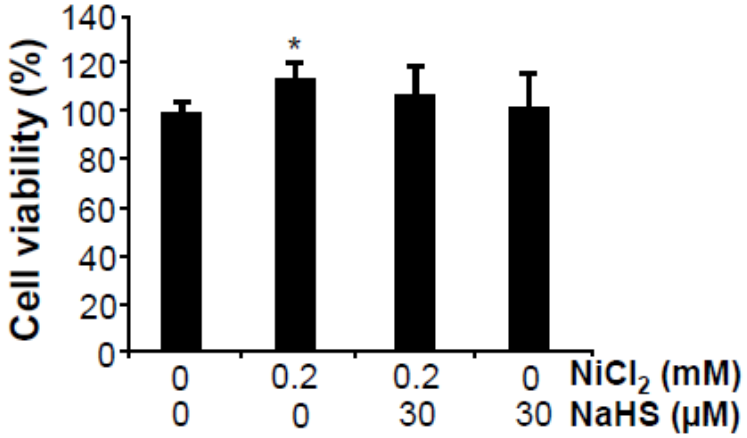

Figure 4.1.2 **The interaction of nickel and H_2S on HF cell viability.** HF cells were incubated with 0.2 mM $NiCl_2$ with and without 30 µM NaHS for 24 hours. Cell viability was measured following treatments by MTT assay. * $p<0.05$ versus control. n = 3

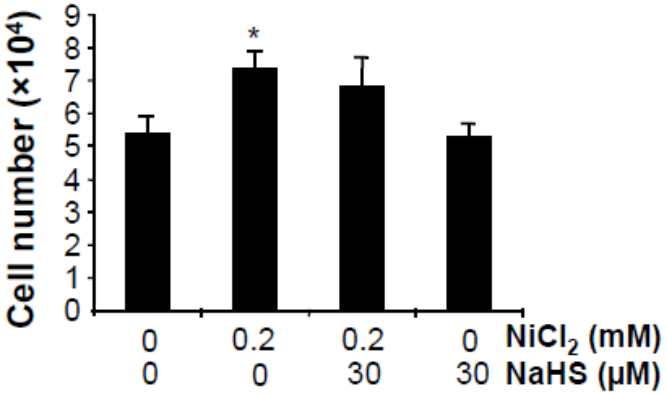

Figure 4.1.3 **Effect of nickel and H_2S on HF cell number.** Equal numbers of cells were seeded and grown. After 24 hours of treatment, cells were trypsinized and counted using hemocytometer. * $p<0.05$ versus control. n =3

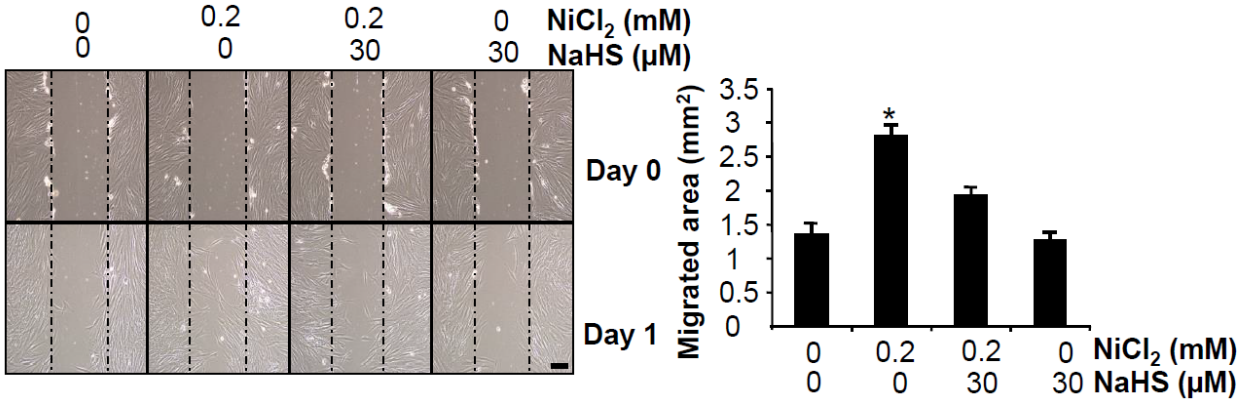

Figure 4.1.4 **Effect of nickel and H_2S on HF cell migration.** HF cells were grown post-confluence, wounding was induced by a single pass with a sterile pipette tip. Serum-free medium and treatments were added for 24 hours. Scale bar: 50 μm *, $p<0.05$ versus all other groups. n = 3

4.2 H_2S reverses nickel-induced fibroblast cell activation

Quiescent HFs have elongated, spindle and flat shapes, and when grow in a crowd, the cells tend to align in parallel. Exposure of HFs to 0.2 mM $NiCl_2$ for 24 hours caused morphological alterations, and the cells became shorter and had higher contractility (figure 4.2.1). Interestingly, co-incubation of HFs with NaHS for 24 hours at 30 μM reversed nickel-altered cell morphology. A marker of fibroblast cell activation is the increased expression of αSMA. Nickel treatment significantly induced the expression of αSMA, as demonstrated by immunofluorescence (figure 4.2.2), western blotting (figure 4.2.3), and real-time PCR (figure 4.2.4). Nickel also dramatically stimulated the mRNA expression of fibronectin, a gene responsible for generating a glycoprotein of the extracellular matrix (figure 4.2.5). Exogenously applied NaHS completely restored the expressions of αSMA and fibronectin induced by nickel, indicating a protective role of H_2S against nickel-induced fibroblast cell activation. By using MEFs isolated from WT mice and CSE-KO mice, we also found that deficiency of CSE worsen nickel-induced expressions of αSMA (figure 4.2.6).

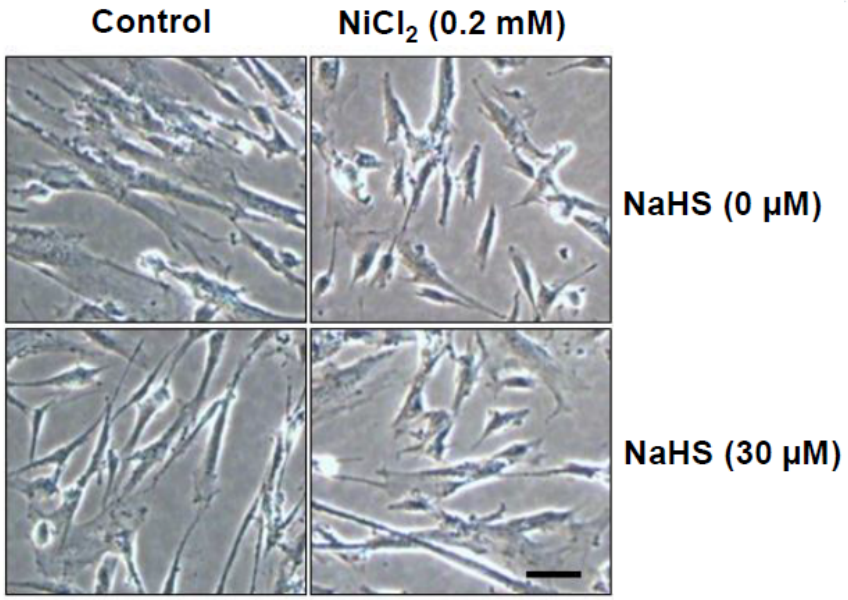

Figure 4.2.1 **Morphology change of HF cells after nickel treatment.** The cells were incubated with 0.2 mM $NiCl_2$ in the presence or absence of NaHS (30 μM) for 24 hours. Scale bar: 20 μm. n = 3

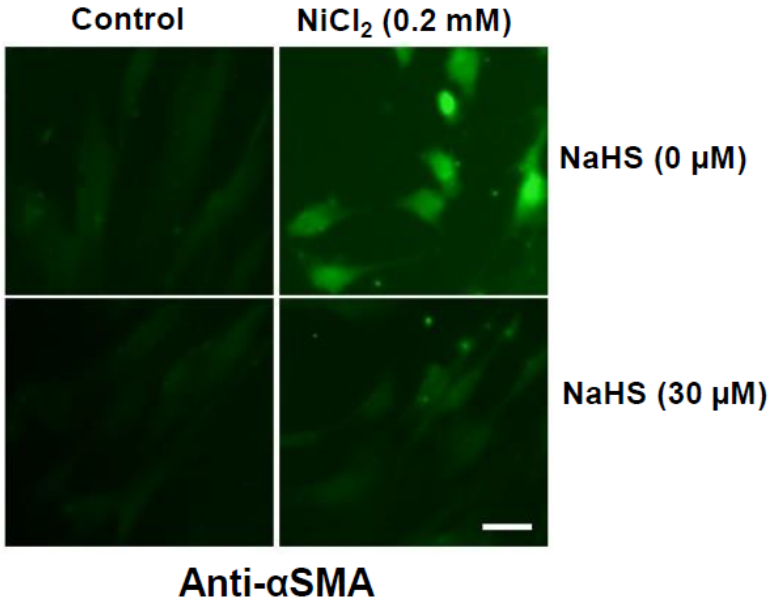

Figure 4.2.2 **αSMA immunostaining in nickel-treated HF cells.** After 24 hours of treatment with 30 μM NaHS and/or 0.2 mM $NiCl_2$, the cells were fixed and subjected to αSMA staining. Scale bar: 20 μm. n =3

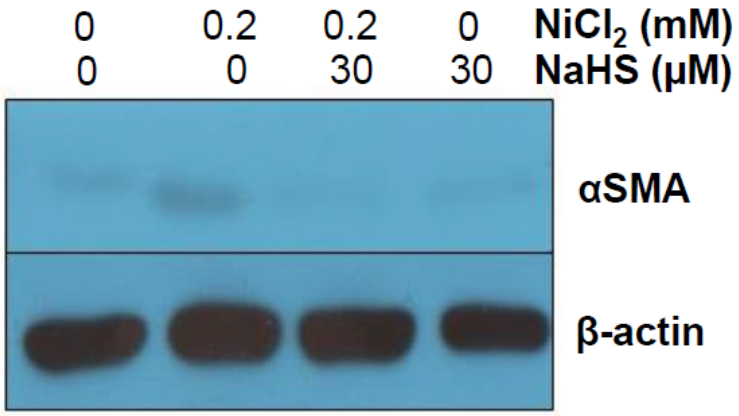

Figure 4.2.3 **Effect of nickel on αSMA protein expression.** The cells were incubated with 0.2 mM $NiCl_2$ in the presence or absence of NaHS (30 μM) for 24 hours. The cells were collected, lysed and protein was isolated in the presence of protease inhibitor followed by western blotting analysis of αSMA and β-actin. n = 3.

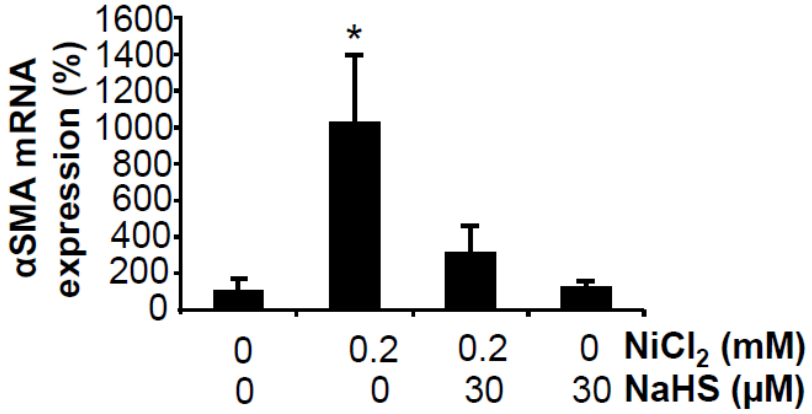

Figure 4.2.4 **Effect of nickel on αSMA mRNA expression.** The HF cells were treated with 0.2 mM $NiCl_2$ in the presence or absence of NaHS (30 μM) for 24 hours. The cells were collected, mRNA was isolated and the expression of αSMA was detected by real-time PCR. *, $p<0.05$ versus all other groups. n = 3

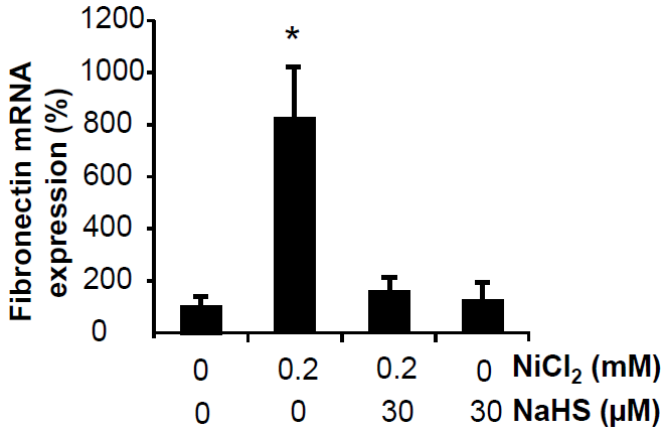

Figure 4.2.5 **Effect of nickel on fibronectin mRNA expression in HF cells.** After 24 hours of treatment with 30 μM NaHS and/or 0.2 mM $NiCl_2$, the cells were collected for detection of mRNA expression by real-time PCR. *, $p<0.05$ versus all other groups. n =3

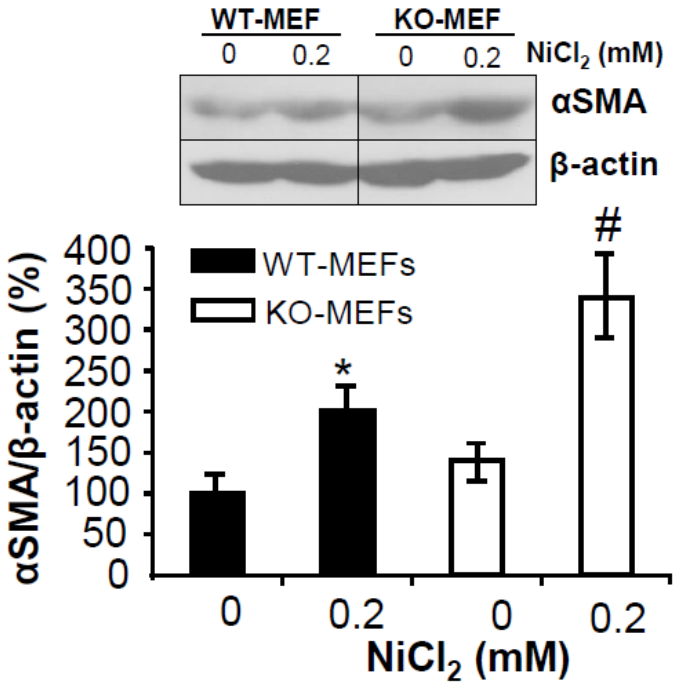

Figure 4.2.6 **Effect of CSE deficiency on αSMA protein expression.** Both WT-MEFs and KO-MEFs were incubated with 0.2 mM $NiCl_2$ for 24 hours. The cells were collected and lysed, and protein expressions were analyzed by western blotting. *, $p<0.05$ versus WT-MEF control; #, $p<0.05$ versus all other groups. n =3

4.3 Nickel inhibits CSE expression and blocks H_2S production

We then examined the effect of nickel on the expressions of three H_2S-generating enzymes. Incubation of HFs with 0.2 mM $NiCl_2$ for 24 hours significantly inhibited CSE protein expression but had no effect on CBS protein expression (figure 4.3.1). The protein expression of 3-MST was not detected in HF cells. CSE mRNA expression was also suppressed by nickel as demonstrated by real-time PCR assay (figure 4.3.2). Furthermore, nickel reduced H_2S production rate by 66.7% in comparison with the control cells, pointing to an inhibitory role of nickel on CSE enzymatic activity (figure 4.3.3). To observe whether nickel can also directly react with H_2S, we mixed $NiCl_2$ and NaHS, which was then exposed to lead acetate paper. As shown in figure 4.3.4, there was clear evidence that nickel scavenged H_2S by direct chemical interaction. All these data indicate that nickel inhibits CSE/H_2S system at different levels, at the transcription level of CSE mRNA, inhibiting CSE enzymatic activity, and directly trapping H_2S.

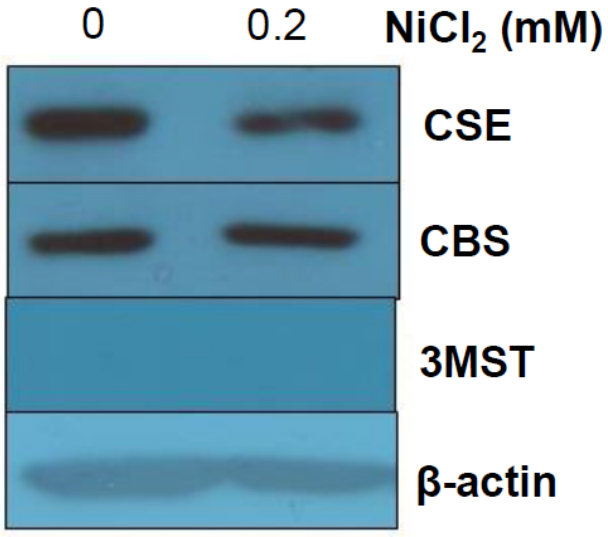

Figure 4.3.1 **Effect of nickel on the protein expression of H_2S-producing enzymes.** HF cells were incubated with 0.2 mM $NiCl_2$ for 24 hours. The cells were collected and protein was isolated followed by western blot analysis of CSE, CBS and 3-MST. β-actin was used as a house keeping gene for normalization. n = 4

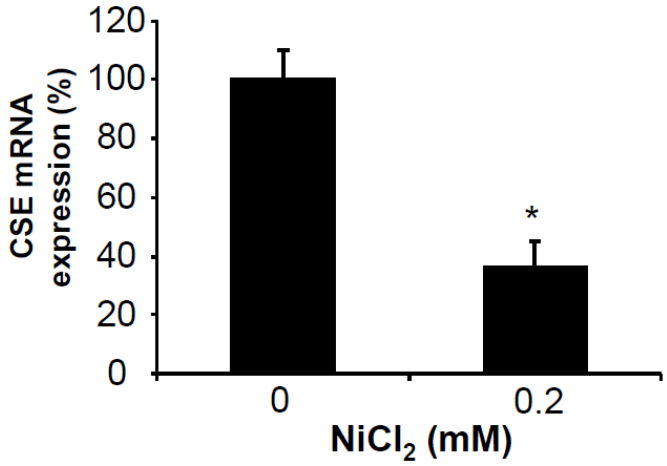

Figure 4.3.2 **Effect of nickel on CSE mRNA expression.** The HF cells were treated with 0.2 mM $NiCl_2$ in the presence or absence of NaHS (30 μM) for 24 hours. The cells were collected, mRNA was isolated and the expression of αSMA was detected by real-time PCR. *, $p<0.05$. n = 4

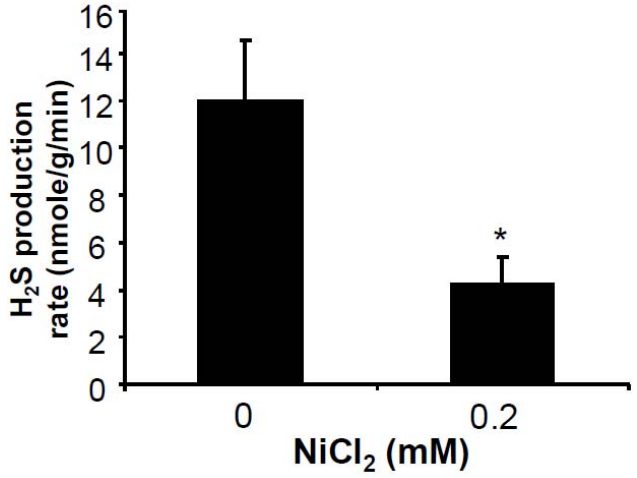

Figure 4.3.3 **Effect of nickel on H_2S production rate in HF cells.** H_2S production rate was measured with methyl blue method. *, $p<0.05$. n =4

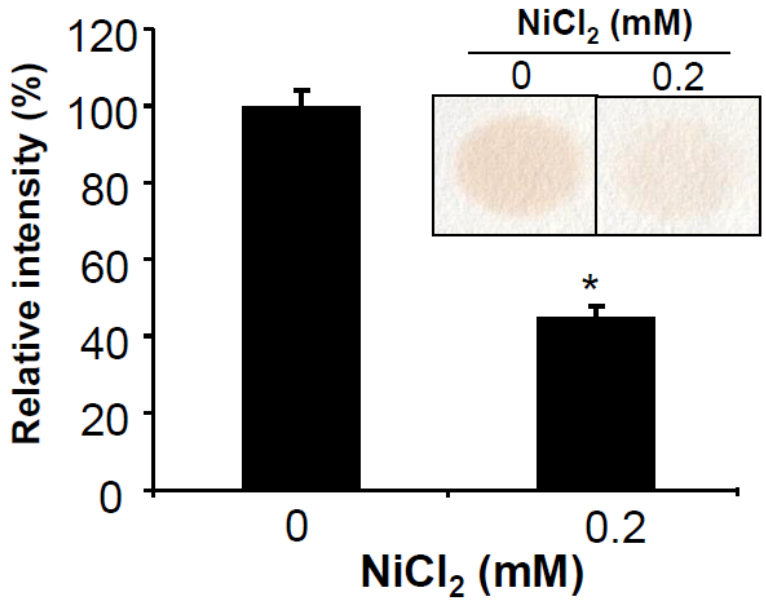

Figure 4.3.4 **The direct interaction between H_2S and nickel.** Direct interaction was measured using acetate lead paper. $NiCl_2$ (0.2 mM) and NaHS (30 μM) were mixed together followed by detection of H_2S release at 37°C for 1 hour. The intensity of the colour was proportional to the amount of H_2S produced. *, $p<0.05$. n =4

4.4 Nickel depletes thiol content and enhances oxidative stress

After 24 hours of incubation with nickel, the intracellular thiol contents were depleted by 45.5%, which was restored to normal level by exogenously applied NaHS. NaHS alone slightly but significantly stimulated thiol contents in HFs (figure 4.4.1). ROS is believed to be an important component in nickel-induced fibroblast cell activation, consequently, we investigated ROS generation in nickel-treated cells. In contrast to the control cells, nickel significantly stimulated the generation of intracellular ROS, which was effectively attenuated by NaHS co-incubation (figure 4.4.2).

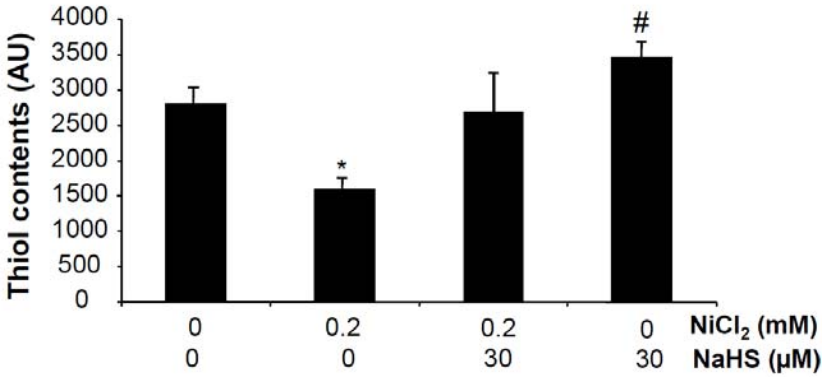

Figure 4.4.1 **Effect of nickel on the thiol content in HF cells.** HFs were incubated with 0.2 mM $NiCl_2$ with or without NaHS (30 μM) for 24 hour, the cells were collected for detection of intracellular thiol contents by detection kit using a proprietary fluorometric detector that reacts with thiol groups to emit a fluorescent signal. (AU: arbitrary unit). *, $p<0.05$ versus all other groups; #, $p<0.05$ versus control. n =4

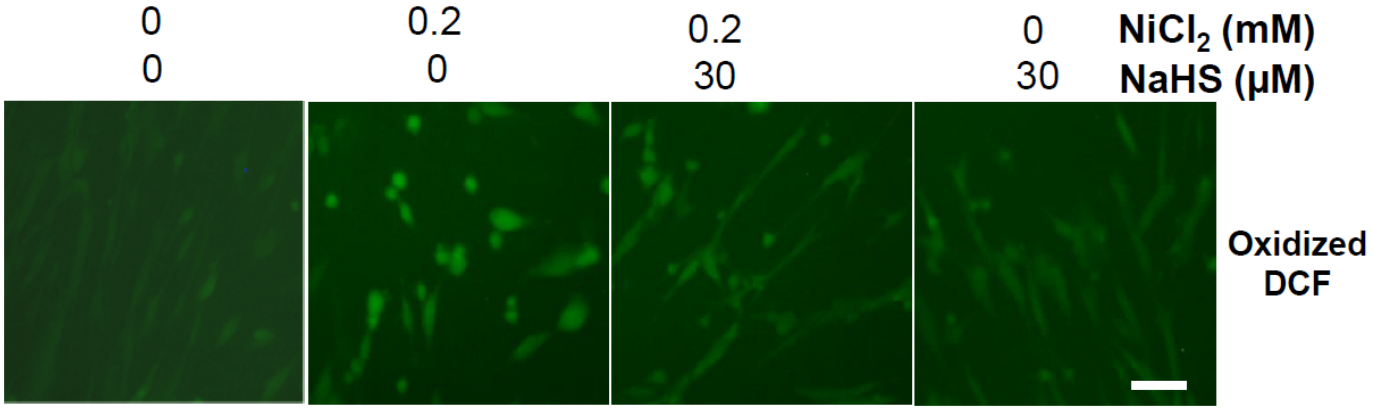

Figure 4.4.2 **Effect of nickel on ROS in HF cells.** After incubation with 0.2 mM $NiCl_2$ with or without NaHS (30 μM) for 24 hour, the cells were incubated with H_2DCFDA for 30 minutes to detect ROS. Scale bar: 20 μm. n = 4

4.5 Involvement of TGFβ1/SMAD1 in nickel-induced fibroblast cell activation

TGFβ1 is one of the major pro-fibrotic cytokines and has been identified as a core pathway of fibrosis. As a result, we next investigated whether the protective effects of H_2S on nickel-induced fibroblast cell activation is related to TGFβ1. Nickel treatment of the cells induced the expressions of TGFβ1 and SMAD1, but had no effect on SMAD2 and SMAD3. Exogenously applied NaHS blocked the stimulatory role of nickel on TGFβ1 and SMAD1 expression (figure 4.5.1). We further validated that TGFβ1 promoted the protein expressions of αSMA and SMAD1 (figure 4.5.2), and the mRNA expression of αSMA (figure 4.5.3) and fibronectin (figure 4.5.4), all of which were partially reversed by NaHS co-incubation.

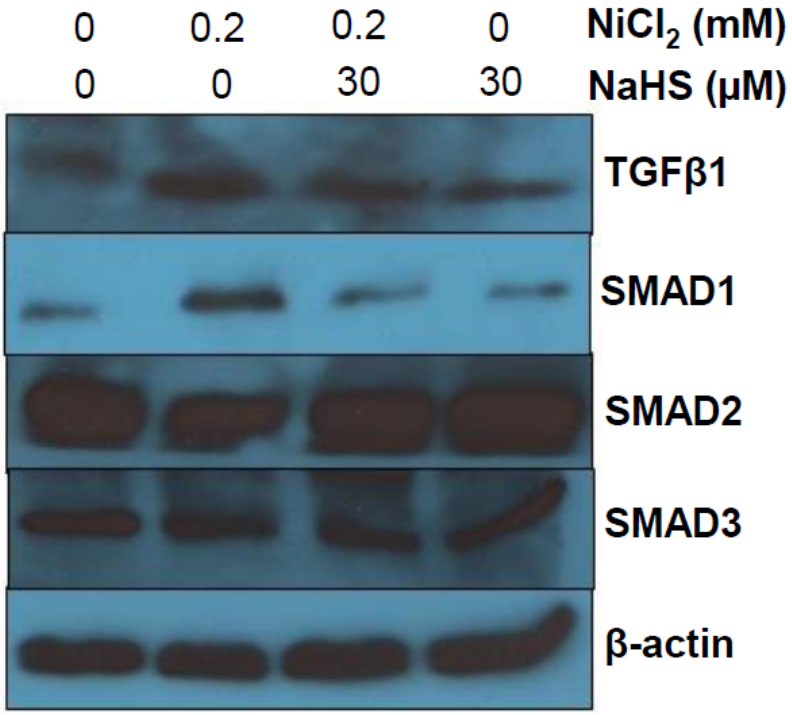

Figure 4.5.1 **Effect of nickel on components of the SMAD pathway in HF cells.** Cells were treated with $NiCl_2$ (0.2 mM) and or NaHS (30 μM) for 24 hours. The cells were collected and protein was isolated followed by western blot analysis of TGFβ1, SMAD1, SMAD2, and SMAD3. β-actin was used for normalization as a house keeping gene. n =3

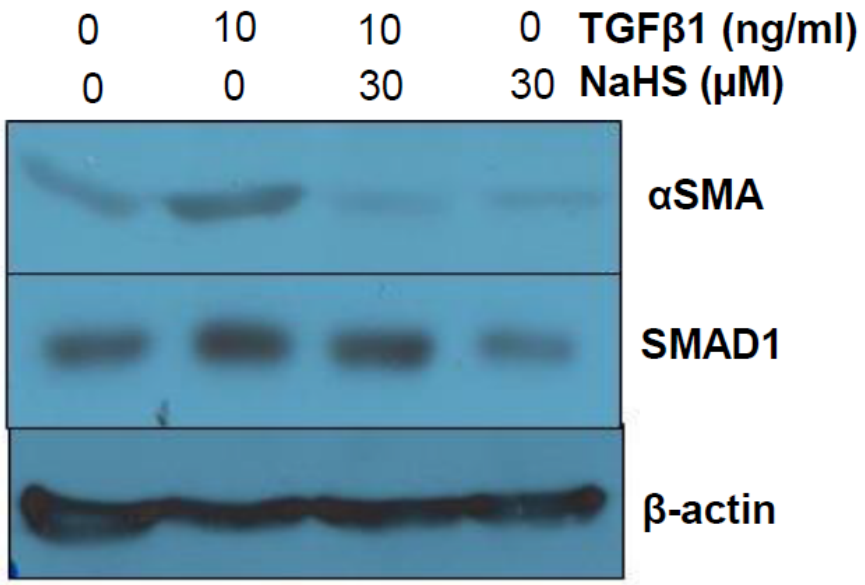

Figure 4.5.2 **Effects of TGFβ1 on αSMA and SMAD1 protein expression in HF cells.** Cells were treated with TGFβ1 (10 ng/mL) and or NaHS (30 μM) for 24 hours. The cells were collected and protein was isolated followed by western blot analysis of αSMA and SMAD1 expression levels. β-actin was used as a house keeping gene for normalization. n =4

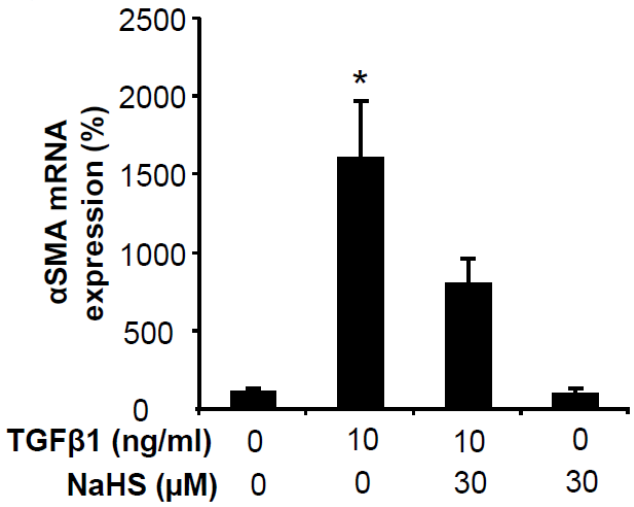

Figure 4.5.3 **Real-time PCR analysis of αSMA mRNA expression in TGFβ1-treated cells.** The HF cells were treated with 10 ng/mL TGFβ1 in the presence or absence of 30 μM NaHS for 24 hours. The cells were collected,

mRNA was isolated and the expression of αSMA was detected by real-time PCR. *, $p<0.05$ versus all other groups. n =3

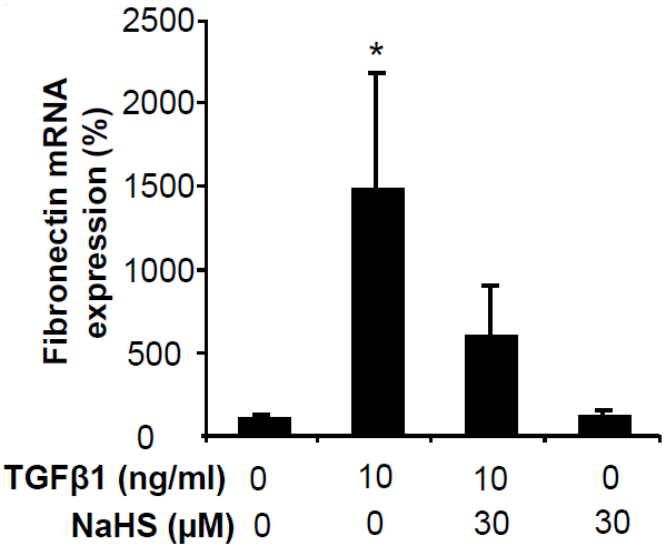

Figure 4.5.4 **Effect of TGFβ1 on fibronectin mRNA expression in HF cells.** After 24 hours of treatment with 30 μM NaHS and/or 10 ng/mL TGFβ1, the cells were collected for detection of fibronectin mRNA expression by real-time PCR. *, $p<0.05$ versus all other groups. n =3

TGFβ1 also dose-dependently induced cell viability, which was not affected by NaHS (figure 4.5.5). In contrast, NaHS blocked the stimulatory role of TGFβ1 on cell migration (figure 4.5.6). TGFβ1, fibronectin and type I collagen had no effect on H_2S production rate and did not chemically react with H_2S (figure 4.5.7 and 4.5.8).

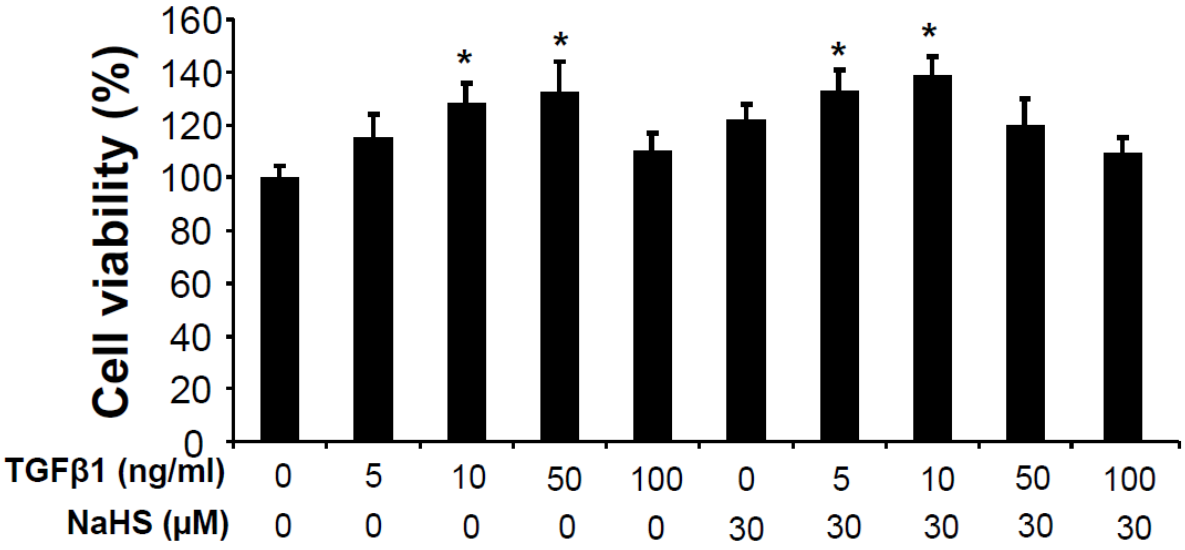

Figure 4.5.5 **Effects of TGFβ1 and NaHS on HF cell viability.** HF cells were incubated with TGFβ1 and NaHS at the indicated concentrations for 24 hours. Cell viability was measured following treatments by MTT assay. * $p<0.05$ versus control. n =4

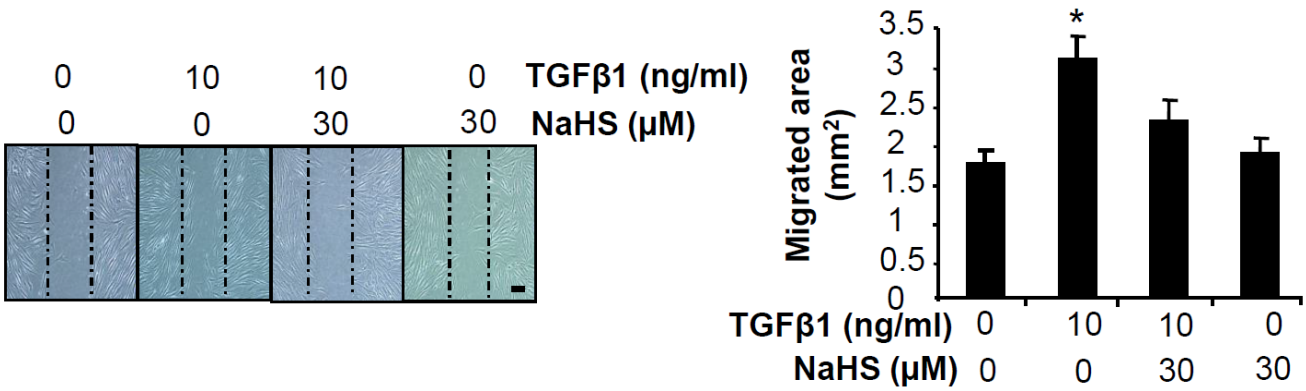

Figure 4.5.6 **Effects of TGFβ1 and NaHS on HF cell migration.** HF cells were grown post-confluence, wounding was induced by a single pass with a pipette tip. Serum –free medium and TGFβ1 (10 ng/mL) and NaHS (30 μM) treatments were added for 24 hours. Scale bar: 50 μm *, $p<0.05$ versus all other groups. n = 3

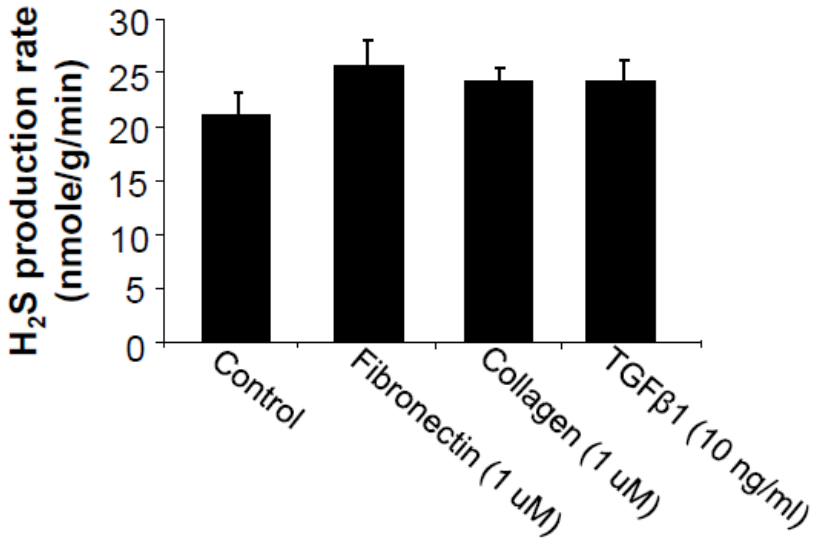

Figure 4.5.7 **Effect of ECM components on H_2S production rate.**. Liver tissues from WT mice were used for measurement of H_2S production rate by methyl blue method. Treatment groups included TGFβ1, fibronectin, and collagen at the noted concentrations. n =4

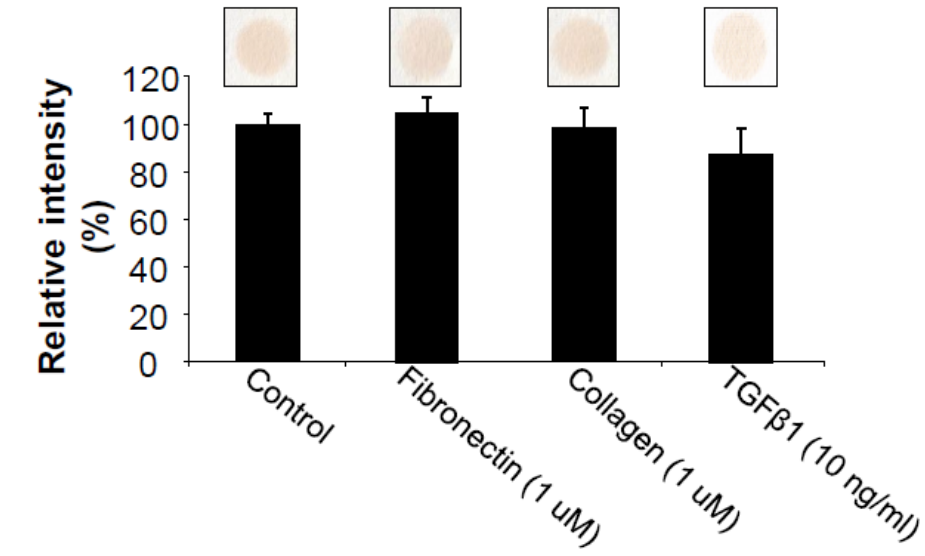

Figure 4.5.8 **Measurement of the direct interaction between H_2S and ECM constituents.** Direct interaction was measured using acetate lead paper. Fibronectin (1 μM), collagen (1μM) or TGFβ1 (10 ng/mL) was mixed with NaHS. Followed by detection of H_2S released at 37°C for 1 hour. n =3

Altered expression and activation of MMPs are often involved in fibroblast cell activation, however we found that the protein expression of MMP2 was not changed by either nickel or H_2S (figure 4.5.9). The three members of MAPK (ERK1/2, p38, and JNK) were also not affected by nickel treatments as seen in figure figure 4.5.10.

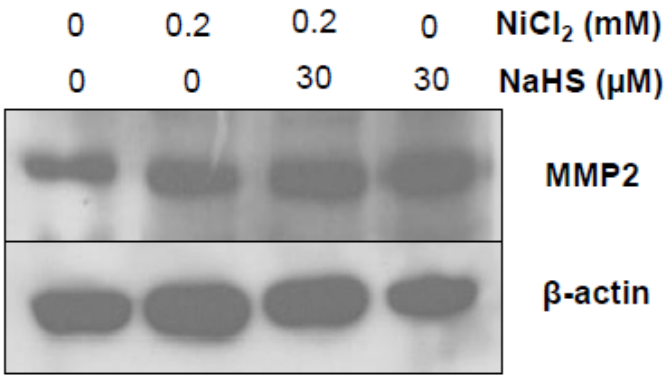

Figure 4.5.9 **Effect of nickel on MMP2 protein expression in HF cells.** The cells were incubated with 0.2 mM $NiCl_2$ and/or 30μM of NaHS for 24 hours. The cells were collected and protein was isolated followed by western blot analysis of MMP2. β-actin was used as a house keeping gene for normalization. n =3

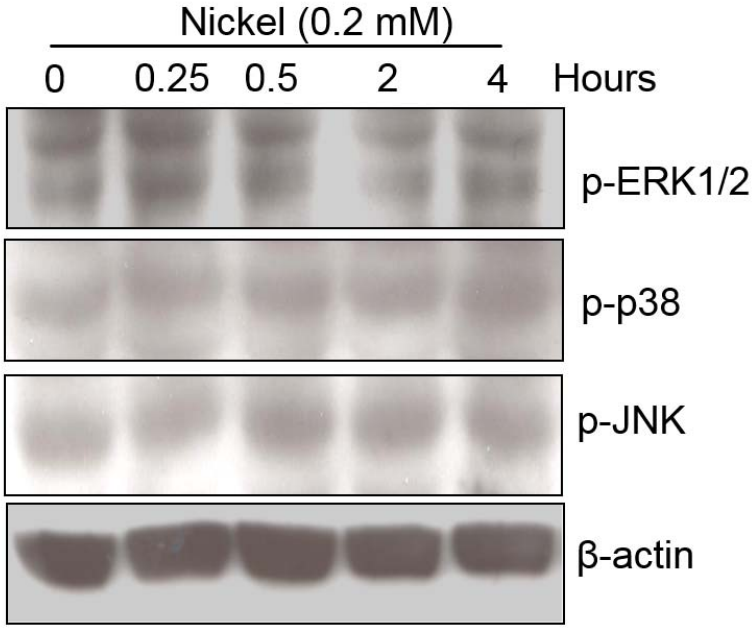

Figure 4.5.10 **Effect of nickel on members of MAPK pathway.** HF cells were incubated with 0.2 mM $NiCl_2$ and at the given times. The cells were collected and protein was isolated followed by western blot analysis of p-ERK 1/2, p-p38, and p-JNK. β-actin was used as a house keeping gene for normalization. n=3

4.6 The interaction of nickel and H_2S in mobilizing intracellular labile zinc and altering Sp1 transactivation

Sp1, a transcription factor with a DNA binding domain composed of three zinc fingers, has been proven to bind to GC-rich motifs in the promoters of both CSE and TGFβ1 genes. To explore whether nickel changes the transactivation of Sp1, Sp1 cDNA was transfected into HFs. As expected, Sp1 overexpression stimulated the protein expressions of both CSE and TGFβ1. The addition of nickel did not change Sp1 expression but differentially alter the regulatory role of Sp1 on CSE and TGFβ1 expression. In the presence of nickel, Sp1 further strengthened TGFβ1 expression but lost its stimulatory role on CSE expression (figure 4.6.1). By using ChIP assay as

shown in figure 4.6.2, we then observed that nickel attenuated the binding of Sp1 with CSE promoter but enhanced the binding of Sp1 with TGFβ1 promoter, which were completely reversed by the supplement of NaHS. NaHS alone had no effect on the interaction of Sp1 with CSE and TGFβ1 promoters. As Sp1 binds directly to DNA via its zinc finger protein motif and nickel can compete with zinc to bind with protein, we then investigated whether nickel would alter intracellular labile zinc. By using a zinc-sensitive fluorophore, FluoZin-3, we observed that nickel incubation of the cells for 1 hour or 24 hour slightly induced the fluorescent intensity of intracellular zinc, although it did not reach statistical significance (figure 4.6.3). The supplement of NaHS showed no effect on intracellular labile zinc.

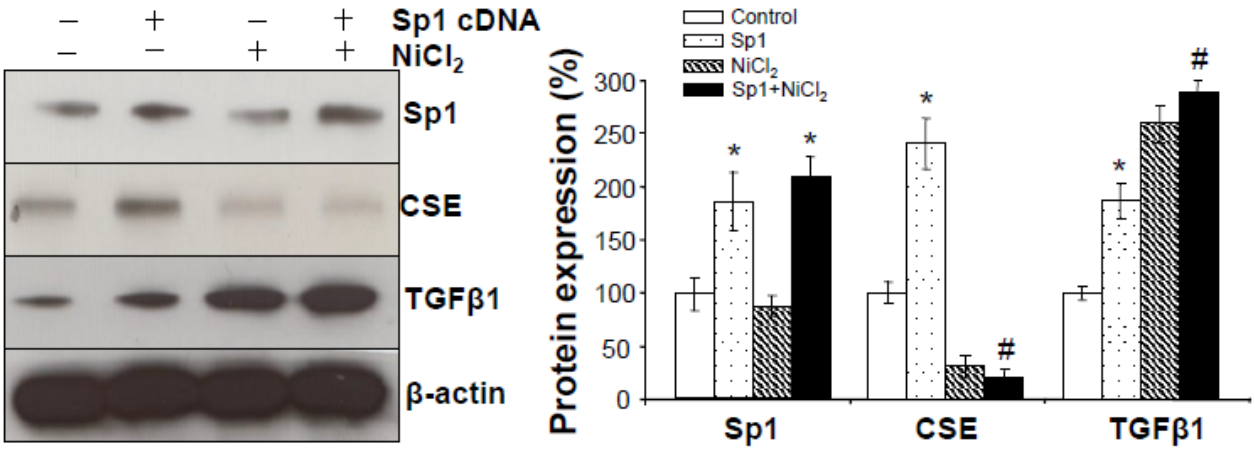

Figure 4.6.1 **Effect of nickel on Sp1, CSE and TGFβ1 protein expression in Sp1-transfected HF cells.** The cells were transfected with Sp1 cDNA for 24 hours, $NiCl_2$ (0.2 mM) was added for another 24 hours. The cells were collected and protein was isolated followed by western blot analysis of Sp1, CSE and TGFβ1. β-actin was used as a house keeping gene for normalization. *, $p<0.05$ versus control or $NiCl_2$ group. #, $p<0.05$ versus Sp1 group. n=3

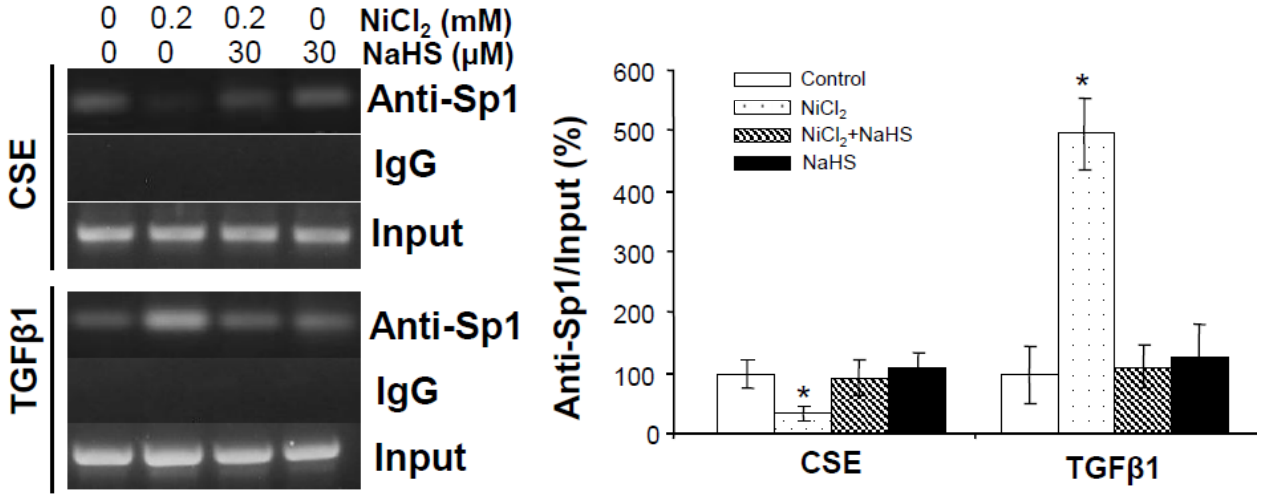

Figure 4.6.2 **ChIP assay analysis of Sp1 binding with CSE and TGFβ1 promotor.** After the cells were treated with $NiCl_2$ (0.2 mM) and/or NaHS (30 μM) for 24 hours, the cells were processed for ChIP assay with the antibody against Sp1. Then the interaction between Sp1 and the promoter was quantitatively analysed by real-time PCR. The binding images were shown in the left panel, and quantitative analysis by real-time PCR as shown in right panel. * $p<0.05$ versus all other groups. n = 3

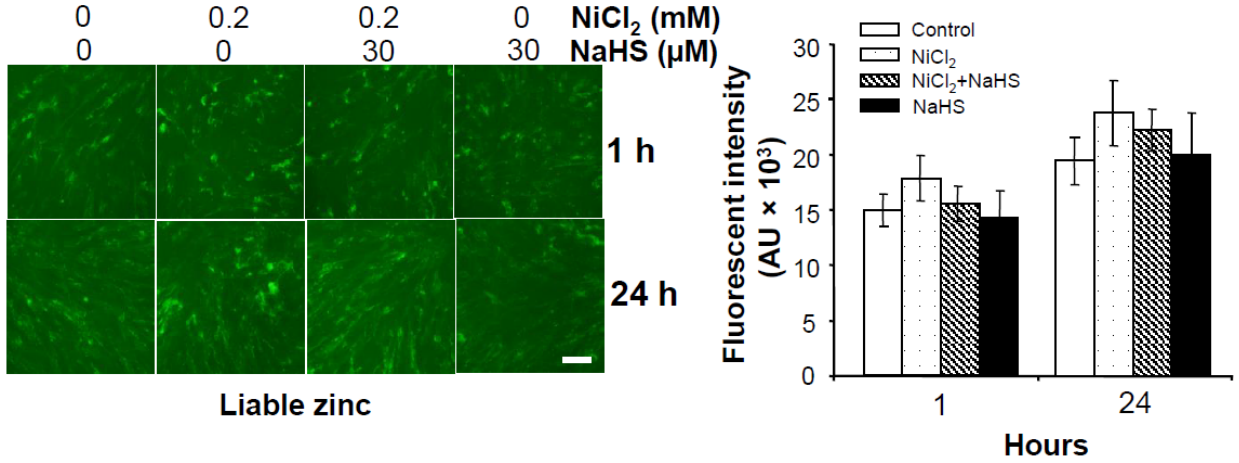

Figure 4.6.3 **Effect of nickel on labile zinc in HF cells.** The cells were treated with 0.2 mM $NiCl_2$ and/or NaHS (30 μM) for 1 hour and 24 hours. Cells were incubated with a zinc-sensitive fluorophore, FluoZin-3 and fluorescence was measured. AU: arbitrary unit Scale bar: 20 μm. n = 3

5 Discussion

Nickel was classified as a toxic compound by the International Agency for Research on Cancer in 1990 (111, 118, 153). Animal and human exposure to nickel lead to abnormal metabolic processes and have increased risk of various organ disorders (153, 154). Bioaccumulation of intracellular nickel can disrupt protein structure and alter enzyme activity by preferentially binding to sulfur-containing groups in specific amino acids (118, 155). Nickel also can crosslink with nucleotides and impair gene replication, transcription and DNA damage repair (111). One of the detrimental effects of nickel is to cause fibrogenesis and tumorigenesis by inducing epithelial-mesenchymal transition (EMT) (156). The present study validated that nickel exposure to human fibroblasts promoted their activation, as evidenced by morphological alterations, increased cell growth, migration, and higher expressions of αSMA and fibronectin, all of which are typical features of EMT and are closely associated with the pathogenesis of tissue fibrosis.

Interestingly, traditionally known as a noxious and toxic gas but now recognized as a new gasotransmitter, H_2S was found to be a target of nickel-induced fibroblast activation. We first found nickel incubation of the cells reduced the mRNA and protein expression of CSE, a major H_2S-generating enzyme in fibroblasts. Although CBS (another H_2S-generating gene) was also expressed in fibroblasts, it seemed that nickel had no effect on CBS expression. Moreover, nickel directly blocked the enzyme activity of CSE with an unknown mechanism. We further confirmed that nickel was able to react with H_2S gas and reduced its release from NaHS solution. All these suggest the inhibitory role of nickel on CSE/H_2S system, pointing to the possibility of the mediation of H_2S in nickel-induced fibroblast activation. Indeed, supplement of H_2S reversed but

complete knockout of CSE enhanced the pro-fibrotic effect of nickel. It is worth noting that numerous studies have demonstrated the protective role of H_2S in the development of fibrosis in lung, liver, kidney, and heart, etc (141, 142). The protective role of H_2S against heavy metal-induced damage have also been reported in many other cell types. H_2S reacted directly with methylmercury to form a less toxic metabolite and attenuated methylmercury-induced neurotoxicity via the inhibition of oxidative stress and protection of mitochondria (157, 158). H_2S attenuated uranium-induced acute nephrotoxicity through oxidative stress and inflammatory response via Nrf2-NF-κB pathways (159). H_2S was also involved in homocysteine-prevented lymphocyte viability loss by cobalt (160).

To investigate the mechanisms underlying H_2S-protected fibroblast cell activation by nickel, we looked at the TGFβ/SMAD pathways. It is well known that TGFβ, through both SMAD-dependent and SMAD-independent mechanisms, is a major driver in promoting the expressions of αSMA and ECM-generating genes, leading to the transformation of fibroblasts into myofibroblasts and excessive accumulation of ECM (67). Nickel increased the expression of TGFβ1 and SMAD1 but had no effect on SMAD2 and SMAD3 as well as the phosphorylation of ERK1/2, p38, and JNK, indicating only TGFβ1/SMAD1 is responsible for the induction of αSMA and fibronectin. We further found that the addition of TGFβ1 stimulated SMAD1 expression following enhanced expression of αSMA and fibronectin. TGFβ1 also induced cell migration. All these effects were blocked by exogenously applied H_2S, suggesting the involvement of CSE/H_2S signal in nickel-promoted TGFβ1/SMAD1 signals. TGFβ1 is also involved in the expression of MMP capable of degrading ECM (100); however our data showed that MMP2 was not affected by either nickel or H_2S. MMPs are a family of zinc-dependent

endopeptidases, it is possible that nickel/H_2S would change MMP2 enzymatic activity by disturbing zinc affinity toward to MMP2 protein, which deserves to be further tested.

Generation of oxidative stress are often reportedly contributed to nickel toxicity and fibrogenesis (161, 162). Here we proved that nickel treatment of cultured cells oxidized dichlorofluorescin to a fluorescent product and induced the level of oxidants in the cells. The higher level of ROS by nickel may be due to the low level of thiol, since nickel significantly lowered the contents of intracellular thiol. Thiol, a sulphydryl (SH) group-containing compound, plays significant roles in defense against oxidative stress (163, 164). Nickel has high affinity to interact with thiols by forming planar complexes. It has been reported that mutation of cysteine 295 in carbon monoxide dehydrogenase-II resulted in the decrease of nickel content and N-acetyl cysteine attenuated nickel-induced production of ROS (165, 166). H_2S was able to increase glutathione level by stimulating the expressions of GSH-synthesizing genes (151). H_2S also reduced the disulfide-bond inside protein by forming S-sulfhydration (167). By competing with nickel to bind with SH-containing thiol compounds, H_2S would eventually diminish the inhibitory role of nickel on the level of total thiols.

Nickel suppressed CSE expression but induced TGFβ1 expression. It is not clear how nickel differentially affects CSE and TGFβ1. Sp1, a transcription factor with three contiguous $Cyseine_2$-$Histidine_2$ zinc finger domains, regulates gene transcription by binding to GC-rich motifs of many promoters (168). We and others demonstrated that both CSE and TGFβ1 acted as downstream genes of Sp1 (35, 85, 86). Nickel had no effect on Sp1 expression, however nickel blocked the stimulatory role of Sp1 on CSE expression and further strengthened the stimulatory of Sp1 on TGFβ1 expression. These effects were attributed to the different role of nickel in altering the interaction of Sp1 with CSE and TGFβ1 promoters. Excess or deficiency of divalent

zinc impairs DNA binding of Sp1 (168, 169). Nickel alters the activities of zinc-containing proteins/enzymes through mobilization of intracellular zinc, which can be further strengthened by chelating zinc, pointing to an important role of zinc in antagonizing the effect of nickel (170). Zinc fingers are reactive towards nickel, with zinc/nickel substitution and the oxidation of cysteine residue(s) in zinc fingers as major reactions (170, 171). So the zinc in Sp1 zinc-finger domains are susceptible to nickel hydrolysis due to the nickel over-accumulation inside the cells. Previous studies have shown that nickel substitution of zinc in Sp1 altered Sp1 binding sequence by changing the conformation of Sp1 DNA-binding domain (169, 171). Although the change of total labile zinc by nickel was not significant, it does not exclude the possibility that individual protein (such as Sp1) can be targeted by nickel to replace zinc or nickel can bind with the protein without affecting zinc crosslink with the protein. Nickel exposure can selectively damage Sp1 interaction with gene promoters, which initiates the pro-fibrotic activity of nickel compounds (169). H_2S S-sulfhydration modification of proteins by forming a –SSH bond with the target cysteine residues, and Sp1 has been reported to be one target for H_2S S-sulfhydration (172). Based on these evidence, it can be speculated that, the supplement of H_2S reverses the effects of nickel on Sp1 transactivation by promoting the structure stability of zinc-finger domains, while reduced level of H_2S due to low expression of CSE promotes the pro-fibrotic effect of nickel.

6 Conclusion

Taken together, our results demonstrated that nickel at low concentration acts as a pro-fibrotic molecule by altering Sp1 transactivation, inhibiting the CSE/H_2S system, activating TGFβ1/SMAD1 pathway, upregulating the expressions of αSMA and fibronectin, and reducing intracellular thiols (Fig. 6.1). Supplement of H_2S donor was sufficient to reverse the stimulatory role of nickel on fibroblast cell activation. Future studies aim at dissecting the role of H_2S in protecting tissue fibrosis by nickel and/or other heavy metals in animals and human.

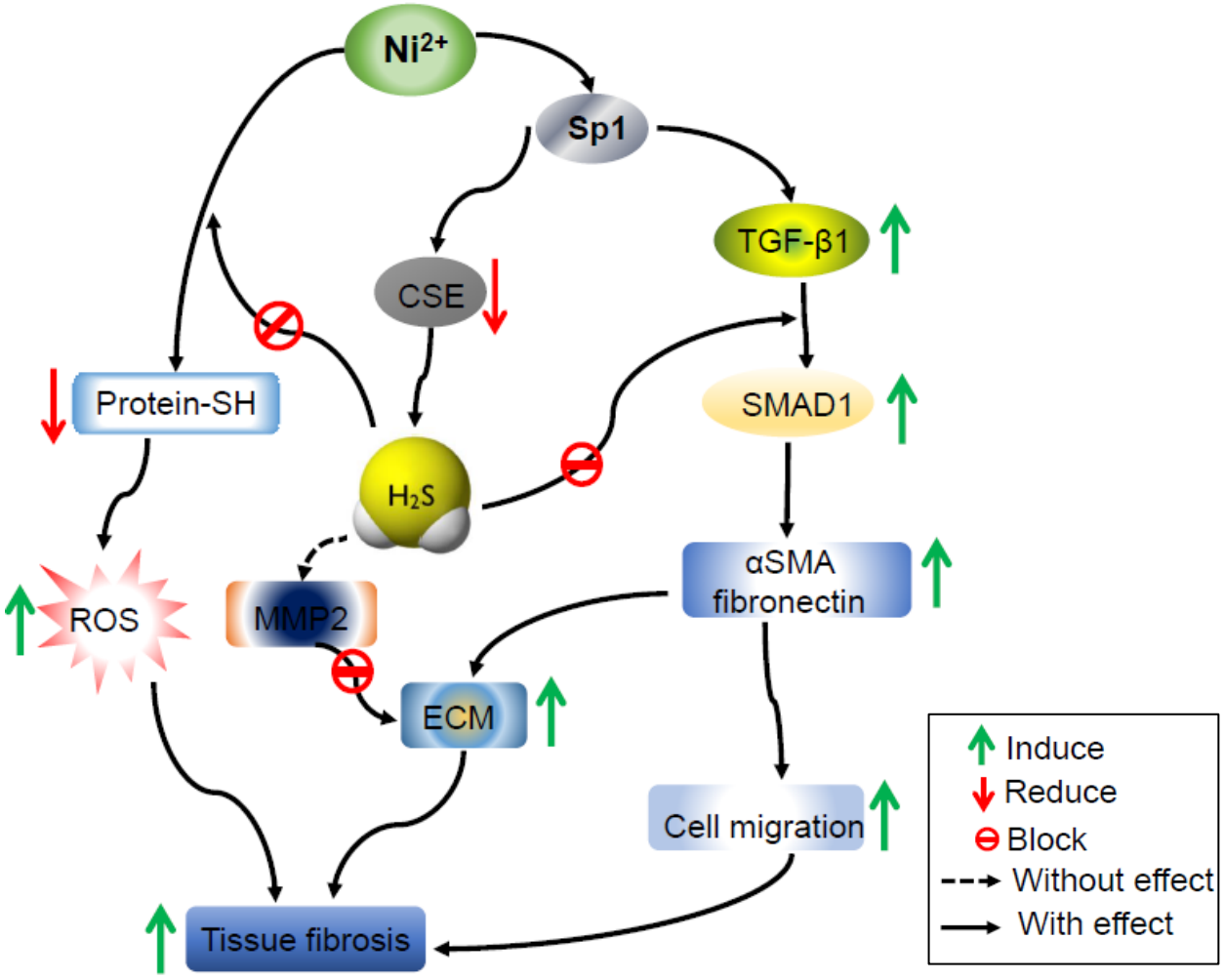

Figure 6.1 **Proposed mechanism underlying H_2S protection against nickel induced fibroblast activation**. Nickel acts as a pro-fibrotic molecule by altering Sp1 transactivation, inhibiting the CSE/H_2S system, activating TGFβ1/SMAD1 pathway, upregulating the expressions of αSMA and fibronectin, inducing cell migration, upregulating ECM production attributing to tissue fibrosis. Nickel also attenuates intracellular thiols consequently increasing ROS leading to damage and tissue fibrosis. Supplement of H_2S donor was able to reverse the stimulatory role of nickel on TGFβ1/SMAD1 activation and protects the cells from the effect of nickel on thiol levels. H_2S demonstrated no effect on MMP2 proteins.

7 Limitation of this study and future work

The present study was undertaken to determine the interaction of H_2S and nickel in fibroblast cells and investigate the potentially protective role of CSE/H_2S system in nickel-induced fibroblast cell activation as well as the underlying mechanism. One limitation of this study is it was only observed in cultured fibroblast cells, whether the results are widely applicable still needs to be tested in other mammalian cells and also in whole animals. The deletion of CSE gene led to significant suppression of endogenous H_2S production and caused hypertension and atherosclerosis in mice. CSE knockout mice would be a good model to further determine the protective role of endogenous H_2S against nickel toxicity.

This study focused on the effects of exogenous H_2S by using a fast-releasing H_2S donor, NaHS. When NaHS is added into the culture media, only one third H_2S is released in seconds and the concentration of remaining H_2S is far less, as expected, within hours. Slow and stable releasing donors for H_2S, such as GYY4137 and sulforaphane, can be further examined.

There are still many gaps in the knowledge base that needs to be filled to fully understand the mechanisms. Further investigation of the molecular mechanisms underlying the roles of CSE/H_2S system in regulating the TGFβ1/SMAD pathway and fibroblast cell activation will facilitate the development of novel therapies for the treatment of tissue fibrosis. In addition to TGFβ1/SMAD signaling, other pathways may also mediate the regulatory role of H_2S in nickel-induced fibroblast activation, which needs to be explored. S-sulfhydration is proposed to be the main mechanism for H_2S signaling. It is worthy of further testing to see whether these downstream molecules act as targets by H_2S for S-sulfhydration.

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